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15679

Relation of Survival Time of Respiratory Gasping Mechanism of the Isolated Mouse Head to Age.

RICHARD K. THOMS AND WILLIAM A. HIESTAND.

*From the School of Pharmacy and the Laboratory of Animal Physiology, Purdue University,
Lafayette, Ind.*

It was shown previously¹ that survival of the "primitive respiratory center" of the rat head is inversely proportional to age of the animal. The method used was the recording of the gasping (mandibular movements) following decapitation. Young rats (under 5 or 6 weeks of age) showed 2 periods of gasping, the initial period being aerobic and the second period anaerobic. Rats older than 5 or 6 weeks were reported to show only the first (aerobic) series. Similar results were later obtained with ischemic heads of rabbits, cats, and dogs.² Work from our

laboratory³ has corroborated these findings, however it has been observed⁴ that occasionally an adult rat may show a few gasps of the second series. Kabat⁵ previously demonstrated a reciprocal relationship between age and resistance to arrest of brain circulation in dogs.

That the sudden change of the newborn mammal from the fetal to the outside environment may be more successfully accomplished a source of anaerobic energy is available to the young. This fact coupled with

¹ Selle, W. A., and Witten, T. A., *Proc. Soc. Exp. Biol. and Med.*, 1941, **47**, 495.

² Selle, W. A., *Proc. Soc. Exp. Biol. and Med.*, 1941, **48**, 417.

³ Hiestand, W. A., Tschirgi, R. D., and Miller, H. R., *Am. J. Physiol.*, 1944, **142**, 153.

⁴ Tschirgi, R. D., personal communication.

⁵ Kabat, J., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 534.

TABLE I.
Showing Relation of Age to Number of Gasps and Survival Time in 144 White Mice.

Age in days	1	3	4	5	6	7	8	9	10	11	12	13	15	19	over 20
No. mice used	8	6	6	6	6	7	10	7	7	6	11	13	2	13	49
Wt in g (avg)	1.7	2.3	2.4	2.7	3.1	3.5	2.6	2.3	4.9	5.3	4.7	4.6	4.6	9.3	27.5
No. gasps 1st ser. (avg)	12.5	9.5	9.8	8.7	8.1	8.1	6.0	11.3	6.4	3.7	6.4	8.0	22.0	7.2	10.9
No. gasps total (avg)	32.1	28.2	20.9	20.3	21.3	26.7	45.7	22.7	43.4	21.5	16.8	22.0	72.0	7.2	10.9
Duration of 1st ser. in sec. (avg)	32.2	19.0	14.7	25.5	13.3	13.1	19.5	24.6	11.6	10.1	13.3	14.5	18.6	16.05	16.05
\pm S.E.	± 2.2	± 1.5	± 2.5	± 4.0	$\pm .97$	$\pm .04$	± 2.5	± 2.9	$\pm .68$	$\pm .99$	± 1.1	± 3.6	± 1.8	± 1.8	$\pm .25$
Total survival time in sec. (avg)	1052.1	1459.2	1326.3	780.0	916.0	576.1	574.3	754.3	354.3	109.0	71.8	64.5	45.0	19.9	16.05
\pm S.E.	± 135.4	± 101.3	± 119.6	± 92.5	± 67.1	± 62.7	± 127.7	± 46.5	± 32.8	± 12.1	± 10.3	± 3.0	± 1.8	± 1.8	$\pm .25$

the ability of the young to withstand anoxia more effectively than the adult has been amply demonstrated.⁶⁻¹⁰

In the present investigation 144 white mice of the Purdue Swiss and Hygienic strains were used, no appreciable difference appearing in the gasping patterns of the 2 strains. Heads were decapitated with a single stroke of a razor blade held vertically in a holder as previously used with rats.³ No gasping results in the mouse unless the cut is made posterior to the fore-legs. Rats will gasp if the cut occurs posterior to the external ear lobe. In both cases the excised head con-

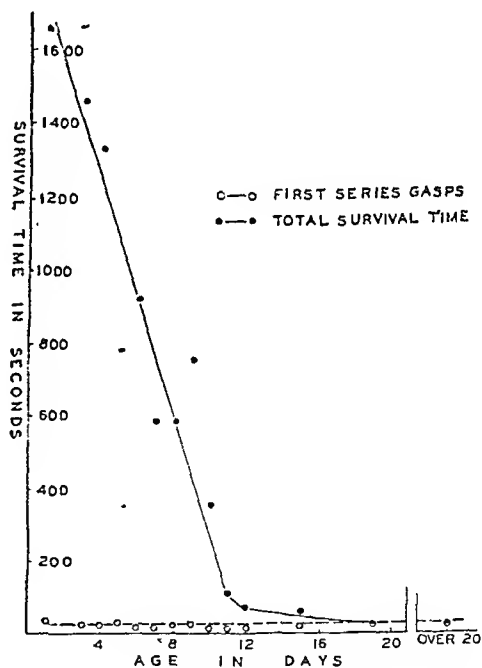


FIG. 1.

Showing survival time in relation to age in 144 mice. Each point represents the average of mice used at a given time.

⁶ Avery, R. C., and Johlin, J. M., *Proc. Soc. Exp. Biol. and Med.*, 1932, **29**, 1184.

⁷ Fazekas, J. F., Alexander, F. A. D., and Himwich, H. E., *Am. J. Physiol.*, 1941, **134**, 281.

⁸ Kabat, H., *Am. J. Physiol.*, 1940, **130**, 588.

⁹ Himwich, H. E., Fazekas, J. F., and Homburger, E., *Endocrinol.*, 1943, **33**, 96.

¹⁰ Himwich, H. E., Bernstein, A. O., Herrlich, H., Chesler, A., and Fazekas, J. F., *Am. J. Physiol.*, 1942, **135**, 387.

tains the medullary centers as well as the carotid reflex areas. Gasps were recorded manually on an electric kymograph traveling one centimeter in 5.540 seconds. Ninety-five mice from the ages of 1 to 19 days and 49 mice older than 20 days were used. In no case did any gasping occur beyond the first (aerobic) series in mice over 19 days of age. This observation holds true for many other mice used in other experiments and not included herein.

Table I and Fig. 1 show the results of this study. It can be seen that the aerobic first series of gasping remains quite constant throughout the life of the mouse indicating

that the availability of the energy material remains little changed. The second or anaerobic series of gasps diminishes until about the 19th day when it is lost forever, thus indicating that the anaerobic energy of the newborn mouse persists for only 19 days.

Conclusions. The survival of the gasping mechanism of decapitated mice, like rats, is inversely related to age. Mice up to 19 days of age show 2 series of gasps, the early or aerobic series remaining quite constant throughout life, the second or anaerobic series gradually diminishing from the maximum in the newborn until it disappears completely at about 19 days of age.

15680

Effects of Urethane (Ethyl Carbamate) on Mitosis.*

M. F. GUYER AND P. E. CLAUS.

From the Department of Zoology, University of Wisconsin, Madison.

The authors¹ showed that transplanted rat carcinoma (Flexner-Jobling carcinoma) was far more susceptible to the lethal effects of X-rays when growing on rats which had been treated with colchicine than when growing on untreated controls. Unable because of war conditions to obtain colchicine to continue their experiments, they resorted to urethane (ethyl carbamate, $C_2H_5OCONH_2$) which was reputed to have much the same effects on mitosis. They found that while urethane does affect mitosis, the result was not the same as that following colchicine. The present study deals with their experiments and observations on this point.

The treatments were standardized to intraperitoneal injections of 1 cc of a 10% aqueous solution of urethane per 100 g of body weight of the young adult rats being treated. This was an anaesthetizing dose which left the animals inert for several

hours. Of the 42 animals treated, 21 were male and 21 female. No significant sexual differences were found. A series of 14 mice were also injected and studied for comparison.

Since the purpose of our study was to determine the effect of urethane on mitosis it was important to select a tissue favorable for study. After some exploration we settled upon the corneal epithelium, recommended by Buschke, Friedenwald and Fleischmann² for mitosis counts and for differentiation of the various phases of mitosis. While the number of division figures vary greatly from the periphery to the center of the cornea, they are approximately the same in all meridians. A band 2 mm wide was cut out along the greatest meridian of the cornea and carefully separated from adherent tissues. After rinsing in 80% alcohol it was placed for an hour each in 95% alcohol, and absolute alcohol, and then stored in cedar oil. After at least an hour in cedar

* Aided by support from McArdle Memorial Laboratory, the Wisconsin Alumni Research Foundation, and the Brittingham Trust Fund.

¹ Guyer, M. F., and Claus, P. E., *Proc. Soc. Exp. Biol. and Med.*, 1939, 42, 565.

² Buschke, W., Friedenwald, J. S., and Fleischmann, W., *Bull. Johns Hopkins Hosp.*, 1943, 72-73, 144.

TABLE I.
Showing Relation of Age to Number of Gasps and Survival Time in 144 White Mice.

Age in days	1	3	4	5	6	7	8	9	10	11	12	15	19	over 20
No. mice used	8	6	6	6	6	7	10	7	7	6	11	2	13	49
Wt in g (avg)	1.7	2.3	2.4	2.7	3.1	3.5	2.6	2.3	4.9	5.3	4.7	4.6	9.3	27.5
No. gasps 1st ser. (avg)	12.5	9.5	9.8	8.7	8.1	8.1	6.0	11.3	6.4	3.7	6.4	8.0	7.2	10.9
No. gasps total (avg)	32.1	28.2	29.9	29.3	21.3	26.7	45.7	22.7	43.4	21.5	16.8	22.0	7.2	10.9
Duration of 1st ser. in sec. (avg)	32.2	19.0	14.5	25.5	13.3	13.1	19.5	24.6	11.6	10.1	13.3	14.5	18.6	16.05
\pm S.E.	± 2.2	± 1.5	± 2.5	± 4.0	$\pm .97$	$\pm .01$	± 2.5	± 2.9	$\pm .68$	$\pm .99$	± 1.1	± 3.6	± 1.8	$\pm .25$
Total survival time in sec. (avg)	1652.1	1439.2	1324.3	780.0	916.0	576.1	574.3	754.3	354.3	109.0	71.8	64.5	19.9	16.05
\pm S.E.	± 135.4	± 101.3	± 119.6	± 92.5	± 67.1	± 62.7	± 157.7	± 46.5	± 32.8	± 12.1	± 10.3	± 35.0	± 1.8	$\pm .25$

the ability of the young to withstand anoxia more effectively than the adult has been amply demonstrated.⁶⁻¹⁰

In the present investigation 144 white mice of the Purdue Swiss and Hygienic strains were used, no appreciable difference appearing in the gasping patterns of the 2 strains. Heads were decapitated with a single stroke of a razor blade held vertically in a holder as previously used with rats.³ No gasping results in the mouse unless the cut is made posterior to the fore-legs. Rats will gasp if the cut occurs posterior to the external ear lobe. In both cases the excised head con-

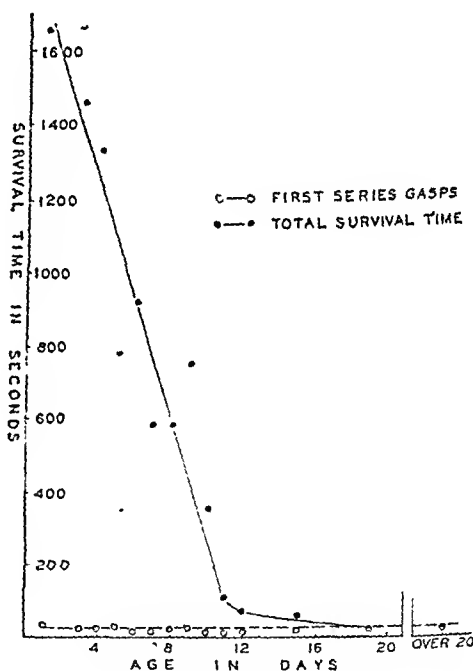


FIG. 1.

Showing survival time in relation to age in 144 mice. Each point represents the average of mice used at a given time.

⁶ Avery, R. C., and Jöhlín, J. M., *Proc. Soc. Exp. Biol. and Med.*, 1932, **29**, 1184.

⁷ Fazekas, J. F., Alexander, F. A. D., and Himwich, H. E., *Am. J. Physiol.*, 1941, **134**, 281.

⁸ Kabat, H., *Am. J. Physiol.*, 1940, **130**, 588.

⁹ Himwich, H. E., Fazekas, J. F., and Homburger, E., *Endocrinol.*, 1943, **33**, 96.

¹⁰ Himwich, H. E., Bernstein, A. O., Herrlich, H., Chesler, A., and Fazekas, J. F., *Am. J. Physiol.*, 1942, **135**, 387.

the distributional mechanism. With urethane, on the contrary, all mitotic stages seem to be largely done away with. Fig. 1 and 2 show how nearly mitotic activities were brought to a standstill.

The graphs are based on corneal sections in which mitotic figures of all phases were counted in every fifth section, with 36 sections inspected for each cornea. Forty-two rats were injected at 9:30 a. m. after which 6 injected and 6 control animals (3 of each sex) were killed at intervals of 4 hours from 10 a. m. one morning till 10 a. m. the next—a sequence of 7 sets in all. For purposes of comparison similar counts were also made on 28 mice.

Fig. 1 represents a series of graphs for the average abundance of each stage, based on sections from the corneas the 21 urethane-treated female rats compared with 21 corneal mitotic counts from untreated controls. The lowermost graph summarizes the general average for all stages taken together. The continuous line of each graph represents the urethane-treated animals, the dotted line, the controls. Horizontal readings indicate the time of day the animals were killed, the up-right column shows the average number of division-figures per section in 108 sections (3×36) of the 3 females killed at any one time. The averages for anaphases and telophase (the 2 uppermost graphs) are based upon a 0 to 1.0 scale in contrast with the others in which the scale ranges from 0 to 50. This is because so many fewer division figures are found in anaphase and telophase than in prophase and metaphase.

The controls, indicated by dotted lines, show a daily rise and fall of the mitotic activities of all stages, with the smallest number of division figures appearing at or a little before 2 a. m., after which it ordinarily rises fairly rapidly till at least 6 a. m. In the treated animals, indicated by the solid lines in the graphs, the disappearance of the several stages is more rapid and it reaches its lowest point several hours earlier than

the normal—by approximately 6 p. m. After remaining stationary or markedly retarded for from 8 to 12 hours, prophases begin to increase in number again. A series of counts on the corneas of the 21 injected and 21 control male rats was also made but inasmuch as it was not essentially different it has not been pictured. Fig. 2 represents a series of graphs made from the corneal cells of 14 urethane-treated and 14 control mice. The stages and designations are the same as for the rats (Fig. 1). It is obvious that response through decline in the number of mitotic figures, and persistence of the inhibition were even more pronounced than in the rat.

It is evident that instead of arresting mitosis in metaphase and holding it there as colchicine does, urethane almost wholly does away with all stages of it for a time. In this respect its effects seem more to resemble those described as following ether, cocaine or ephedrin.

In subsequent experiments with urethane we were surprised to find that it not only affects mitosis but causes pulmonary tumors¹ in well over half (75 out of 86) of our experimental animals. The effect is evidently the same as that reported by Nettleship and Henshaw² for this drug on mice.

Summary. Intraperitoneal injections of urethane (1 cc of a 10% aqueous solution per 100 g of body weight) almost wholly does away with all stages of mitosis for a period of 8 to 12 hours, judging from counts of mitotic figures in sections of the corneal epithelium from rats and mice. In a later experiment, lung tumors of adenomatous type appeared in 75 of 86 animals treated with urethane, but what, if any, connection there is between the interference of this drug with mitosis and its causation of lung tumors is not evident.

¹ Report in press, *Cancer Research*.

² Nettleship, A., and Henshaw, P. S., *J. Nat. Canc. Inst.*, 1943, 4, 309.

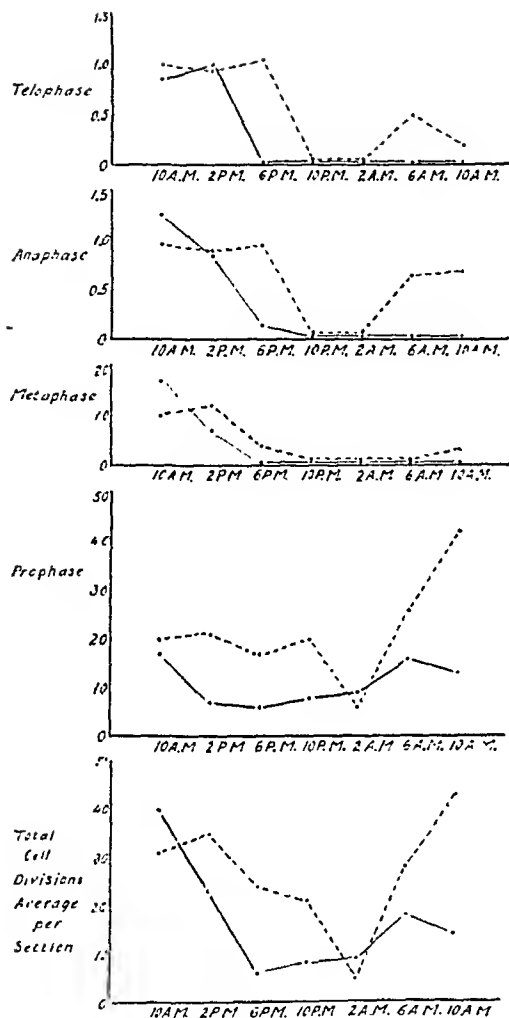


FIG. 1.

Graphs based on counts from the corneal cells of 42 female rats, showing the relative frequencies of the several mitotic stages in individuals killed at 4-hour intervals from 10 a. m. to 10 a. m. The continuous lines indicate mitotic phases in the 21 urethane-treated animals; the broken lines, the phases in the 21 controls. Counts for each phase in both experimental and control animals were made on 3 different individuals. The lowermost series represents the general average for all stages together.

oil the strips were passed successively through absolute alcohol, 95% alcohol, 70% alcohol, and distilled water, remaining for at least 15 minutes in each. They were then stained for 20 minutes in a 1:5 dilution of Harris' modification of Delafield's hematoxylin, rinsed in distilled water, differentiated in

1% HCl until deep red, and then left in ammonia water (0.1%) until blue. No counterstain was used. The strips were then dehydrated in the usual manner and cleared in cedar oil.

For sectioning, the tissues were mounted in paraffin and cut 6 μ thick meridionally of the cornea. According to the study of Buschke, Friedenwald and Fleischmann already cited, one in every 250 cells of the 2 basal layers should, on the average, be found in mitosis. While the range from eye to eye of the same individual is small the variation from animal to animal may be considerable.

The action of urethane on cell-division does not resemble closely that of colchicine. Although delaying mitosis for from 15 to 25 hours, colchicine permits accumulation and persistence of the metaphase stage, with actual continuance of chromosome-divisions. The interference appears to be mainly with

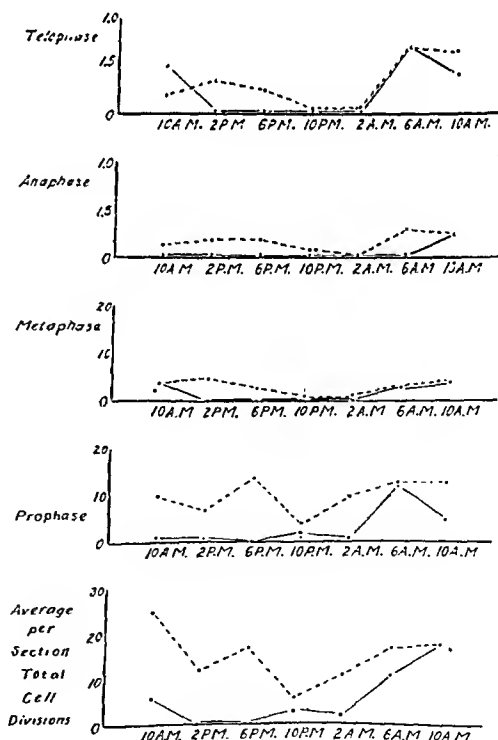


FIG. 2.

Graphs for 28 mice (14 urethane-treated, 14 controls), mixed sexes: counts for each phase made on 2 individuals, otherwise same as Fig. 1.

TABLE I.

Age of Culture, Duration of Guinea Pig Passage and Resistance to Streptomycin of Tubercle Bacilli Isolated from Patients by Direct Culture and by Guinea Pig Inoculation.

Case	Direct culture		Culture from guinea pig*		
	Age of culture, days	Resistance to streptomycin†	Days in guinea pig	Age of culture, days	Resistance to streptomycin†
1‡	102	0.15	42	60	0.15
2‡	161	0.15	67	94	0.15
3	31	100.0	64	42	100.0
4	24	>2000.0	22	55	500.0
5	114	25.0	71	43	25.0
6	181	25.0	67	114	25.0
7	34	>2000.0	68	30	>2000.0
8	114	10.0	63	50	10.0
9	24	>2000.0	61	37	>2000.0
10	74	0.08	93	32	0.31
11	70	>2000.0	58	33	>2000.0
12	98	1500.0	65	33	>2000.0
13	95	25.0	76	135	25.0
14	112	0.15	60	52	0.15

* Inoculated the same days as direct cultures were made with a portion of the same material.

† Greatest concentration of streptomycin allowing growth in micrograms per milliliter of medium.

‡ Untreated patients.

ment has been carried out for several months.

It is evident from the data presented in Table I that cultures of tubercle bacilli from patients receiving streptomycin retain their resistance to the drug even after residence in guinea pigs for 10 weeks or more and subsequent maintenance on glycerinated egg yolk agar for many weeks.

In another experiment each of 14 guinea pigs was inoculated subcutaneously with 0.1 mg of tubercle bacilli found to be resistant

to at least 2,000 μ g of streptomycin per milliliter of medium. Cultures isolated from 9 of these that died after periods of 61 to 164 days of infection were found still to be resistant to at least 2,000 μ g. In all 14 animals there was gross and microscopic evidence of progressive tuberculosis indistinguishable from that seen in a similar group of guinea pigs inoculated with a sensitive strain from the same patient.

15682

Uniformity of Size of Bacteriophage Particles.

A. D. HERSHEY, FRANCES KIMURA, AND J. BRONFENBRENNER.

From the Department of Bacteriology and Immunology, Washington University School of Medicine, St. Louis, Mo.

As measured by the sintered glass boundary method of Northrop and Anson, Kalmanson and Bronfenbrenner¹ found the apparent diffusion coefficient of coliphage PC (hereafter called T2K) to be about 1.7×10^{-7} cm²/sec, corresponding to an equivalent spherical di-

ameter of about 16 m μ . By fractional ultrafiltration, a very small fraction of the phage was obtained which behaved in the same measurement like particles having a diameter of about 4 m μ .

Immunological analysis² revealed a re-

¹ Kalmanson, G. M., and Bronfenbrenner, J., *J. Gen. Physiol.*, 1939, 23, 203.

² Hershey, A. D., Kalmanson, G. M., and Bronfenbrenner, J., *J. Immunol.*, 1943, 46, 281.

Persistence of Resistance of Tubercle Bacilli to Streptomycin During Passage Through Guinea Pigs.

ALFRED G. KARLSON, WILLIAM H. FELDMAN, AND H. C. HINSHAW.

From the Division of Experimental Medicine, Mayo Foundation, and the Division of Medicine, Mayo Clinic, Rochester, Minn.

Youmans and associates¹ have reported that tubercle bacilli from 8 of 12 patients treated with streptomycin had a resistance to this drug 500 to 1,000 times as great as that of cultures taken before treatment with streptomycin. We have studied cultures from tuberculous patients before and during the course of treatment with streptomycin and also have observed this phenomenon. In some instances cultures of tubercle bacilli have shown a resistance to more than 10,000 times the concentration of streptomycin compared to those obtained prior to treatment.

Preliminary observations indicate that resistance to streptomycin may persist after repeated subculture and storage in the refrigerator. We present herewith our findings that tubercle bacilli isolated from guinea pigs inoculated with sputum, urine or material obtained on gastric lavage from tuberculous patients who were being treated with streptomycin are as resistant as cultures isolated directly from the same materials.

Methods. Specimens of sputum, urine or gastric washings were cultured in 4 tubes of glycerinated egg yolk agar and also inoculated into 2 guinea pigs. Cultures were observed weekly and when growth occurred one tube was selected for determination of resistance to streptomycin. The spleens of the guinea pigs were cultured on the egg yolk agar when the animals died or when they were killed in 8 to 9 weeks. The tests for resistance were done in a manner modified from that used by Youmans and his associates. Two-fold dilutions of streptomycin* ranging from 2,000 to 0.04 μg per milliliter of medium were made in Vorwald's

modification of Proskauer and Beck's medium containing 10% horse plasma. This was dispensed in 5 ml amounts into 150 by 15 mm test tubes. The inoculum was prepared by grinding in a mortar and suspending a portion of the culture in Proskauer and Beck's medium.

The suspension of tubercle bacilli was made to contain approximately 1 mg of bacteria per milliliter as determined by comparison with a standard made by use of the Hopkins tube. Each tube of medium was inoculated with 0.1 ml of suspension or approximately 0.1 mg of tubercle bacilli. We have found that the inoculum may vary within relatively wide limits without any variation being reflected in the results. Duplicate tests of tubercle bacilli with a known resistance to streptomycin have given the same results when 0.2, 0.1, 0.05 or 0.025 mg of inoculum was used. After incubation at 37°C for 14 days the resistance to streptomycin was recorded as the greatest concentration that permitted visible growth. When duplicate tests were made or tests were repeated the results occasionally varied to the next higher or lower concentrations. The apparent large differences as in cases 4 and 12 in Table I are inherent in a 2-fold dilution method.

Results. A comparison of resistance to streptomycin between tubercle bacilli cultured directly from sputum, urine or material removed by gastric lavage and those isolated after passage through guinea pigs is given in Table I. Cultures from untreated tuberculous patients usually are resistant to only 0.08 to 0.62 μg of streptomycin per milliliter of medium and the majority have a resistance of 0.15 μg . Our information is not complete but our present data indicate that resistance of tubercle bacilli from patients being treated with streptomycin is not apparent until treat-

¹ Youmans, G. P., Williston, Elizabeth H., Feldman, W. H., and Hinshaw, H. C., *Proc. Staff Meet., Mayo Clin.*, 1946, **21**, 126.

* Furnished through the courtesy of Merck and Co., Rahway, N.J.

TABLE I.
Diffusion Coefficients of T1 and T2K in Agar.

Material	Time (days)	Phage per ml	$D_1 \times 10^8$	$D_2 \times 10^8$	D_2/D_1
Whole T1 + Whole T2K	7	ca. 10^7	1.6	0.98	0.61
	14		0.82	0.53	0.65
	28		0.61	0.44	0.72
	43		0.69	0.50	0.72
Whole T1 + Ultrafiltered T2K	14	ca. 10^5	1.9	1.4	0.74

D_1 = diffusion coefficient of T1.

D_2 = diffusion coefficient of T2K.

served as reference.

For analysis of the contents of the diffusion cell, the agar was cut into segments with a fine stretched wire, beginning from the phage-free end of the column, the latter being expelled from the tube without backward movements by means of the plunger formed by anchoring a wire handle in the paraffin plug. The segments, collected in numbered weighing bottles, were weighed, and their length and position with reference to the boundary fixed by summing the weights. The phage content of the segments was determined by suspending the agar in broth by aspiration with a pipette, and plating after suitable dilution. The recovery of both phages under the conditions of the experiments was quantitative.

Owing to the nature of the analytic method, and to the slow rate of diffusion, only the data for the region of low phage concentration proved useful for the calculation of diffusion coefficients. This disadvantage, as compared with optical methods of analysis in the diffusion of purified proteins for instance, was more than offset by the advantages of being able to use very dilute solutions, and to make analysis from the region of 10^{-1} down to about 10^{-5} times the initial concentration of phage.

For this region, the distribution of phage about the boundary, after correction for the lengths of the individual segments (by graphical integration of the ideal diffusion curve), conformed nearly but not quite to that expected for ideal diffusion. The deviations were systematic, and might be attributed to heterogeneity of the phage, to the curvature of the boundary, or to seepage of

fluid along the walls of the tube.

The pertinent results of 2 experiments are summarized in Table I. It will be seen that the method clearly distinguishes T1 and T2K in mixture, the latter diffusing more slowly. The diffusion coefficients, calculated for the largest distance from the boundary at which dependable analysis could be made, vary from 0.61 to 1.9×10^{-8} cm²/sec for T1, and from 0.44 to 1.4×10^{-8} for T2K. The variation must arise either from errors in locating the boundary, or to differences in the quality of the 2 preparations of agar gel, since the relative coefficients of the 2 phages vary only from 0.61 to 0.74. Since this ratio is not altered by ultrafiltration of T2K, we evidently failed to obtain any fractionation with respect to size of particles of this phage by this method.

The actual size of the particles cannot be estimated from these data, owing to the impeding effect of the gel on diffusion. It is of some interest to note the magnitude of this effect. For T2K, the diffusion coefficient of 0.5×10^{-8} corresponds to an equivalent spherical diameter of about 500 m μ , calculated for diffusion in water. This is about 6 times the probable true diameter,⁸ as if the particles were diffusing freely in the agar gel only about one-sixth of the time allowed. On the other hand, the relative sizes estimated from these data, namely T1 about two-thirds the diameter of T2K, is in good agreement with the corresponding X-ray sensitive volumes⁷ and electron-micrographs.⁸

Other experiments using this same method, in which a second ultrafiltrate of T2K was compared with whole T1, and an ultrafiltrate

markedly high combining power of the phage for neutralizing antibody, about 4600 molecules of antibody per lytic unit of phage. This may be taken as evidence that the preparations of Kalmanson and Bronfenbrenner,¹ weighing about 10^{-13} mg per lytic unit, consisted of remarkably pure antigen derived from the phage: The weight 10^{-13} mg is equivalent to about 30 particles of 16 $m\mu$ diameter, and since the measurement of effective antigenic surface seemed to give independent support for this assumption, it was concluded that the total number of phage particles, each measuring 16 $m\mu$ in diameter, was about 30 times higher than that indicated by the lytic titre.²

Northrop,³ both by diffusion and centrifugal analysis, estimated a diameter of about 10 $m\mu$ for a staphylococcal phage in dilute solution (in concentrated solution it behaved like particles 100 $m\mu$ in diameter). A few phages, namely S13 and C13, have a similar small size (8 to 20 $m\mu$) by either filtration or centrifugal analysis.⁴ Otherwise, and particularly for phages belonging to the serological group (group 11 of Burnet⁵) homologous with T2K, much larger sizes have been found by all workers. For example, phage WLL has a diameter of 50 to 75 $m\mu$ by filtration, and 60 $m\mu$ by sedimentation.⁴ T2K itself has a diameter of about 50 $m\mu$ estimated from the ratio of weight to lytic titre of the best preparations,¹ and T2 (a variant of T2K now known as T2L⁶) has a diameter of about 50 $m\mu$ from the X-ray sensitive volume,⁷ and measures about $65 \times 80 m\mu^8$ or $80 \times 100 m\mu^9$ in electron-micrographs. In addition, approximate one to one correspondence between lytic titre and particle count revealed by means of the electron-microscope,⁸

contradicts our conclusion² regarding the infectivity of the particles.

In view of this unsatisfactory state of affairs,⁴ we began several years ago some additional measurements of the size and uniformity of phage T2K. We have only now been able to correlate our results in a fairly satisfactory way.

We have used 3 methods: diffusion through agar, centrifugation, and diffusion across the sintered glass boundary. In all 3 methods, another coliphage, T1, has been used as reference, the 2 phages being counted separately in mixtures by plating on suitable indicator strains of bacteria each sensitive to only one of the phages.

Diffusion Through Agar. Diffusion through agar was studied as follows. Suitable quantities of melted 3% washed agar in distilled water, and the phages in broth, were mixed at 45°C to give a 0.6% agar solution containing about 10^7 particles each of T1 and T2K per ml. The phages consisted of filtered broth lysates diluted about 1:1000. At the same time a similar solution was prepared containing broth without added phage. The boundary was set up by allowing 10 ml of the phage-free solution to solidify in the lower end of a length of glass tubing (16 mm internal diameter) fitted with rubber stoppers, and then adding an equal volume of the phage-containing solution. After this had solidified, a layer of melted paraffin about 2 cm long was pipetted directly onto the agar surface. This formed a plug serving to minimize mechanical movements of the gel during diffusion, which was allowed to proceed at 5°C in the horizontal direction.

Ultrafiltrates were prepared by passing broth lysates through a collodion membrane of sufficient density to reduce the lytic titre by the factor 10,000.¹ The diffusion measurements were conducted with these fractions in the way already described, except that the ultrafiltrates were incorporated into the agar gel without further dilution, yielding a titer of about 10^5 per ml, and the corresponding phage-free solution contained the same ultrafiltrate inactivated by heating, instead of broth. In these experiments also, unfractionated T1 admixed in equal titer

³ Northrop, J. H., *J. Gen. Physiol.*, 1938, **21**, 335.

⁴ Elford, W. J., in *Handbuch d. Virusforschung*, Doerr and Hallauer, Editors, Vienna, 1939, p. 126.

⁵ Burnet, F. M., *J. Path. and Bact.*, 1933, **36**, 307.

⁶ Hershey, A. D., *Genetics*, 1946, **31**, 620.

⁷ Luria, S. E., and Exner, F. M., *Proc. Nat. Acad. Sci.*, 1941, **27**, 370.

⁸ Luria, S. E., Delbrück, M., and Anderson, T. F., *J. Bact.*, 1943, **46**, 57.

⁹ Hook, A. E., Beard, D., Taylor, A. R., Sharp, D. G., and Beard, J. W., *J. Biol. Chem.*, 1946, **165**, 241.

Since particles of this size would not be sedimentable under the conditions of the centrifugations described above, it is evident that we are not here measuring diffusion, and this conclusion is confirmed by the failure to distinguish T1 and T2K in the same mixture. Probably what we are measuring is actual circulation of fluid through the boundary.

A repetition of the experiments with phage propagated in synthetic medium nevertheless confirmed the earlier results, namely, that whole lysate diluted in synthetic medium passed the boundary at an appreciably slower rate than undiluted ultrafiltered phage, which passed at essentially the same rate as phage in broth. This result is evidently inconsistent with the conclusions reached above.

It was noticed, however, that the synthetic medium used in these experiments¹ was supersaturated with respect to magnesium ammonium phosphate, and eventually deposited crystals on standing in the refrigerator. Since no crystals formed in ultrafiltrates of lysed cultures prepared in the same medium, it was suspected that the difference in the rate of passage through the glass boundary might be due to the clogging of the porous plate by crystal formation, which might be expected to occur preferentially within the pores of the glass. The following experiment seems to show that this is what happens.

Parallel measurements of the rate of passage through the glass boundary were made of the same stock of phage T2K diluted respectively in a "saturated" and a "supersaturated" medium. The supersaturated medium was prepared by mixing the 2 stock solutions¹ composing the medium, and placing the mixture at 5°C for about 12 hours, at which time crystallization had not yet begun. The saturated medium was prepared from the same materials, but was stored in the refrigerator for several days, or as long as necessary, until crystallization was complete, and filtered. Two experiments were done, switching the cells used for the measurement of saturated and supersaturated solutions, and cleaning the cells between runs with chromic-sulfuric acid mixture. The results, expressed as equivalent spherical di-

ameters calculated from the apparent diffusion coefficients, were as follows: in saturated medium, 6.8 and 9.8 $m\mu$, and in supersaturated medium, 22.2' and 24.4 $m\mu$. This difference is clearly an artifact, and very likely has the explanation we have suggested.

Discussion. As a result of the experiments described in this paper, it must be concluded that the unit particles of the phages T1 and T2K in diluted lysates are of very large size. They are probably identical with those identified in undiluted lysates by electron-micrography,^{8,9} analytical centrifugation,⁹ and other means.^{4,7} There is no evidence that these particles are heterogeneous in size, or that they disaggregate appreciably on dilution. The previous conclusion,² based partly on immunological evidence, that lysates are composed of antigenically similar particles about 30 times more numerous than indicated by the lytic titer, has also to be withdrawn. The infectivity of the phage particles must be very nearly perfect, as has been found also by other means.⁸

The very large antibody absorbing capacity² of bacteriophage T2K, which is equally great for several members of the same serological group,⁶ may now be accounted for in terms of the analytical data for T2 reported by Hook and co-workers.⁹ These data, which refer to morphologically and centrifugally homogeneous preparations, indicate a considerably larger size than any of the previous estimates. The electron-micrographs show particles $80 \times 100 m\mu$ or $86 \times 113 m\mu$, exclusive of tail, depending on the source of material. From the sedimentation constant (which, however, unaccountably decreases at low concentrations) one estimates an equivalent spherical diameter of 60 to 130 $m\mu$ for the densities 1.5 to 1.1 respectively. From the weight per lytic unit of 10^{-15} gm, and assuming perfect infectivity, one estimates a spherical diameter between 100 and 150 $m\mu$, depending on the density and hydration assumed. A sphere of diameter of 100 $m\mu$, plus a tail of $20 \times 120 m\mu$,⁸ provide a primary physical surface of about $4 \times 10^{-10} \text{ cm}^2$, sufficient to accommodate 4000 molecules of antibody each requiring an area of 10^{-13} cm^2 . This

TABLE II.
Sedimentation of Fractions of T2K in Mixture with T1.

Source of material	No. of Exp.	% sedimented			
		T1		T2K	
		Mean	Extremes	Mean	Extremes
Whole broth lysates	7	14	4-28	84	61-99
Ultrafiltered T2K + whole T1	6	13	5-34	91	76-99
Supernates, T2K	3			87	69-97

of T1 with whole T2K, yielded less presentable data, but nevertheless confirmed our principal conclusion, that ultrafiltration does not affect the speed of diffusion of these 2 phages relative to each other. The 3 ultrafiltrates examined in this way were prepared with 3 different collodion filters.

Centrifugation. The sedimentation experiments were also essentially qualitative, but yielded unequivocal results owing again to the use of a second phage as standard of reference. The method follows.

Three ml volumes of the mixed phages in broth (about 10^5 lytic units per ml) were spun for one hour at 18,000 r.p.m. in 6 ml tubes in an international centrifuge equipped with "multispeed" angle head. The mean distance from the axis of rotation was about 6.5 cm, corresponding to a centrifugal field of 23,500 g. When spun in a cool room, the temperature in the tubes did not exceed 45°C, and the phage could be recovered quantitatively by mixing the contents of the tube at the end of the run. Supernates were sampled by withdrawing 0.5 ml with a pipet from the upper portion of the fluid. Assays of these samples were compared with the same materials uncentrifuged, and in many of the experiments an assay was made also of the mixed residue in the bottom of the tube, as a parallel check against inactivation. Table II shows the results of several experiments with T1 and T2K from lysates prepared in broth, with ultrafiltrates of T2K prepared with 2 different collodion filters, and with T2K from supernates of previous centrifugations. We have omitted from these data a few discrepant experiments, in which both phages failed to sediment appreciably, since this happened just as frequently with unfractionated as with fractionated phage,

and can safely be attributed to accidents of convection.¹⁰ Excluding these, the results show clearly that:

(1) T2K sediments rather completely under the conditions of these experiments, whereas admixed T1 is left largely in the supernate.

(2) The few particles of T2K passed by 3% collodion membranes sediment just as rapidly in the ultrafiltrate as whole phage diluted to the same titer in broth. This conclusion must be qualified somewhat, owing to inaccuracies of the method, but it is clear that we have failed to obtain a fraction of T2K containing particles anywhere near as slowly sedimenting as those of T1.

(3) T2K in supernates from which the bulk of the phage has been removed by previous centrifugation, is not less easily sedimentable than the unfractionated material. In another experiment not shown in the table, 3 successive centrifugations of an undiluted lysate threw down respectively 98.5, 96, and 97% of the residual unsedimented phage.

We conclude that the particles of T2K in dilute solution are essentially homogeneous in size, or at any rate all are appreciably larger or denser than those of T1.

Passage through the sintered glass boundary. In a single experiment, carried out as described by Kalmanson and Bronfenbrenner,¹ it was found that whole T2K in broth, and ultrafiltered T2K from a broth lysate, passed through the sintered glass boundary into broth, or into heated ultrafiltrate, respectively, at an identical rate corresponding to a spherical diameter of about 4 m μ . In both cells, the admixed T1 and T2K passed in precisely the same proportion.

¹⁰ Stanley, W. M., *J. Exp. Med.*, 1944, 79, 267.

Effect of 2,3-Dimercaptopropanol (BAL) on Acute Poisoning by Tervalent and Quinquevalent Antimonial Drugs.*

JAMES F. GAMMILL, CHESTER M. SOUTHAM, AND H. B. VAN DYKE.

From the Department of Pharmacology, College of Physicians and Surgeons, Columbia University, New York.

Among the numerous reports on the experimental use of BAL to combat poisoning by inorganic and organic metallic compounds, the investigation of Braun, Lusky and Calvery¹ is the only one describing work with antimonial drugs. These authors concluded from their work with rabbits that "With the administration of BAL, the tolerance of the rabbits to lethal doses of compounds of antimony was increased more than 50%." Our experiments were all performed with young rats in which we were able to demonstrate a protective action of BAL against only one antimonial, tartar emetic. The lethal action of 4 other antimonial drugs, including Fuadin and Neostam which were also used by Braun, Lusky and Calvery, either was not affected or was increased by the administration of BAL.

Methods. All the experiments were performed with young albino rats (Sherman strain) usually weighing about 75 g (range 55-140 g). A total of 210 female and 885 male rats was used. Each group, usually of 10 animals, if treated with BAL, had a companion group of 10 of the same weight range and sex receiving only the antimonial drug. The dose of antimonial chosen was sufficient to kill 60-100% of animals in the preliminary experiments with single doses. If the BAL had no clearly favorable influence on the lethal action of the drug, the dose of the latter was reduced to determine whether there was an appreciable beneficial or deleterious action of BAL. In some experiments, smaller doses of an antimonial drug and of BAL were administered repeatedly.

In every experiment the antimonial and the BAL solutions were administered by dif-

ferent routes. When an aqueous solution of the antimonial drug was injected intraperitoneally, BAL in oil, according to the formula of Eagle,² was given subcutaneously after dilution of the solution by peanut oil. Solutions of BAL in oil were unsatisfactory for repeated injection over a period of days owing to the accumulation of unabsorbed oil. In later experiments aqueous solutions of drug and of BAL were injected by the same routes as those used by Stocken and Thompson³ in experiments with sodium arsenite; the antimonial drug was injected intramuscularly and freshly-prepared aqueous BAL solution was given intraperitoneally. The antimonial drugs tested were tartar emetic (antimony potassium tartrate), Fuadin[†] (NaSb-bis-pyrocatechindisulfonate of Na), Neostibosan[†] (Sb complex of *p*-aminophenylstibonic acid, *p*-acetylaminophenylstibonic acid, antimonie acid and diethylamine), Neostam[†] (nitrogen-glucoside of sodium *p*-aminophenylstibonate), and Stibanose[†] (Solustibosan or monohydroxytriethylammonium salt of 2,3,antimonie ester of sodium hexonate). The antimony of tartar emetic and of Fuadin is tervalent, whereas it is quinquevalent in the other 3 compounds. Stibanose is of such low toxicity that the large volume of concentrated solution required for a lethal effect could not be injected intramuscularly. The aqueous solutions of Neostam and Neostibosan for intramuscular administration contained respectively 20 g and 30 g of drug per 100 ml of solution.

² Eagle, H., *J. Fcn. Dis. Inf.*, 1946, **27**, 114.

³ Stocken, L. A., and Thompson, R. H. S., *Biochem. J.*, 1946, **40**, 535.

[†] Kindly supplied by the Sterling-Winthrop Research Institute.

[‡] Kindly supplied by the Burroughs-Wellcome Company.

* This investigation was aided by a grant from the Sterling-Winthrop Research Institute.

¹ Braun, H. A., Lusky, L. M., and Calvery, H. O., *J. Pharm. and Exp. Therap., Suppl.*, 1946, **87**, 119.

size is therefore compatible with the observed combining ratio of 4600 molecules per lytic unit,² a correlation which also holds with fair exactness for antigens as diverse in size and shape as ovalbumin, thyroglobulin, hemocyanin,¹¹ and tobacco mosaic virus.¹²

These experiments have some bearing on the question of the utility of the porous plate boundary method of Northrop and Anson,¹³ for the measurement of coefficients of diffusion. In this method, the estimated coefficient is directly proportional to the fraction of the solute diffusing in unit time. Under our conditions, the rate of flow of fluid through the boundary, measured in terms of the amount of bacteriophage passed, is about 0.01 ml per hour, equivalent to a diffusion coefficient of about 4×10^{-7} cm²/sec. This is of the same order as the diffusion coefficient of hemoglobin.¹³ It is evident that this rate of flow would not seriously affect the measurement of diffusion of substances diffusing 5 to 10 times faster than hemoglobin, e.g., of the HCl or other electrolyte used in calibration. For proteins of the size of hemoglobin, the error would be about 100% for solutions having a viscosity near that of water. Actually, protein solutions of sufficient concentration for analysis of the diffusate have a considerably higher viscosity, and the data of Northrop and Anson¹³ suggest that under their conditions, the flow of fluid is negligible compared with the diffusion of hemoglobin, which means that the flow is at least several times slower than in our experiments. Bacteriophage T2,

taken to be a sphere of about 90 m μ in diameter, would have a diffusion coefficient of about 2.7×10^{-8} cm²/sec in water, and would pass through glass discs such as we have used at a rate equivalent to about 6×10^{-4} ml of solution per hour by diffusion alone. Observed amounts in excess of this would provide a very accurate measure of the speed of flow of liquid. A small amount of bacteriophage included in the solution whose diffusion is to be measured would thus permit a very simple correction for what is probably the principal source of error in the use of this method.

Summary. Neither fractional ultrafiltration nor fractional centrifugation have revealed any heterogeneity of size among the particles of the bacteriophage T2K (formerly called PC). The methods used for the measurement of size, namely, diffusion through agar and sedimentation in an angle centrifuge, were essentially qualitative, but the experimental arrangements were such as to yield unequivocal results.

It must be concluded therefore that this bacteriophage has a minimal particle diameter of 60 m μ or more, as has been found previously for unfractionated phages of the same group by a variety of methods.

It is necessary also to withdraw the conclusion previously reached that this bacteriophage has a low infectivity for susceptible bacteria.

An explanation is suggested for some anomalous results previously obtained in attempts to measure the diffusion of bacteriophage through the porous glass boundary. The use of bacteriophage to measure the rate of flow of fluid as a means of control in the application of this method to other materials is described.

¹¹ Hershey, A. D., *J. Immunol.*, 1941, **42**, 485.

¹² Schramm, G., and Friederich-Freksa, H., *Z. physiol. Chem.*, 1940, **270**, 233.

¹³ Northrop, J. H., and Anson, M. L., *J. Gen. Physiol.*, 1929, **12**, 543.

Six experiments were performed with the other trivalent antimonial, Fuadin. In one experiment with BAL in oil the mortality in the drug control group was identical with that in the BAL-treated group. In all the other experiments, whether aqueous BAL or BAL in oil was administered, BAL shortened greatly the period of survival and the rate of recovery from toxic or LD_{40-60} doses of Fuadin. This is clearly shown in the experiment of Fig. 4A. A control experiment demonstrating the efficacy of aqueous BAL in arsenite poisoning is illustrated in Fig. 4B, confirming the report of Stocken and Thompson.³

Two experiments with aqueous solutions of Neostam and BAL demonstrated that treatment with BAL causes an earlier death (at a dosage of 600 mg of Neostam per kg) or increases the mortality rate (at a dosage of 450 mg of Neostam per kg). The latter experiment is summarized graphically in Fig. 5. Neostibosan was the quinquivalent

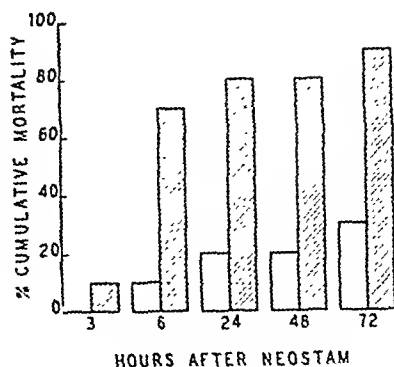


FIG. 5.

Lethal action of BAL in rats receiving a single intramuscular injection of 450 mg of Neostam per kg. BAL, as a single intraperitoneal injection of 0.1 ml of a saturated aqueous solution per 100 g (about 60 mg per kg), was given 5 minutes after the injection of Neostam. No further deaths occurred between the 72nd and the 240th hours. Each group consisted of 10 rats.

White columns: Neostam alone.

Shaded columns: Neostam and BAL.

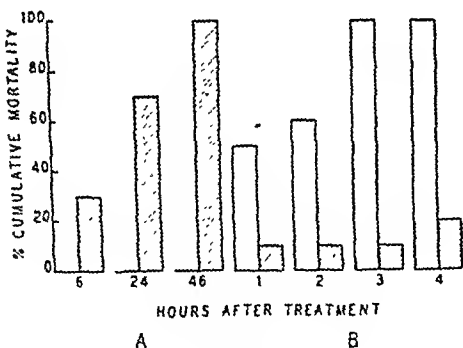


FIG. 4.

A. Lethal effect of treatment with BAL in rats receiving a single intramuscular dose of 250 mg of Fuadin per kg. Without BAL-treatment no rats died within 96 hours. In the group of rats receiving 0.1 ml of saturated aqueous solution of BAL per 100 g (about 60 mg per kg), 70% died within 24 hours and all were dead within 46 hours. Each group consisted of 10 rats.

B. Protective action of BAL in arsenite poisoning. Each rat received, per kilogram, an intramuscular injection of 18 mg of As_2O_3 neutralized by NaOH. The intraperitoneal injection of saturated aqueous solution of BAL was given 10 minutes after the arsenite (0.1 ml per 100 g); a second dose of BAL (0.05 ml per 100 g) was injected 4 hours later. There were no deaths after 4 hours. Each group consisted of 10 rats.

White columns: arsenite alone.

Shaded columns: Fuadin and BAL (A) or arsenite and BAL (B).

antimonial used in 6 satisfactory experiments in which single or repeated daily injections were made. Subcutaneous BAL in oil or intraperitoneal aqueous BAL increased the mortality rate. For example, after 1500 mg of Neostibosan per kg intramuscularly, 50% of the BAL-treated animals and none of the drug controls were dead after 3 hours; at the end of the experiment, 40% of the drug control group were dead and 70% of the group receiving intraperitoneal BAL as well as Neostibosan had succumbed. The low toxicity of Stibanose required intraperitoneal injections (e.g., a single dose of 7000 mg per kg caused only 20% mortality) and the BAL was administered in oil subcutaneously. The effect of BAL (20 mg per kg as a single dose or 2 doses 4 hours apart) apparently was to increase the mortality rate, but this effect was not shown to be significant.

Discussion. The findings here reported, in which rats were the experimental subjects, demonstrate that BAL is of real value in lessening the mortality rate of animals receiving single or repeated doses of tartar emetic. This conclusion is in agreement with that reached by Braun, Lusky and Calvery, whose experiments were all performed with rabbits receiving intramuscular injections of

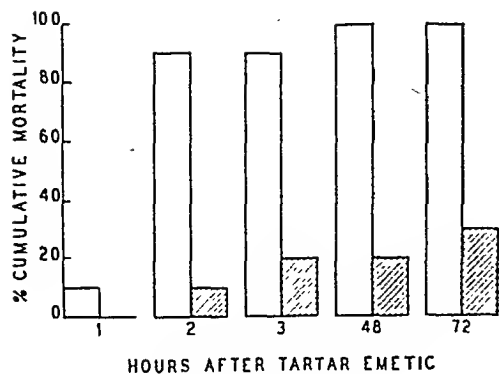


FIG. 1.

Effect of BAL-treatment in the rat on the lethal action of one intramuscular injection of 60 mg of tartar emetic per kg. A saturated aqueous solution of BAL (about 6%) was injected intraperitoneally 5 minutes after tartar emetic in a dose of 0.1 ml per 100 g (about 60 mg per kg). Half of this dose of BAL was repeated 3, 6, and 24 hours after the tartar emetic. One drug-control rat died between the 3rd and the 11th hours of the experiment; no BAL-treated rats died after 72 hours. Each group consisted of 10 rats.

White columns: drug alone.

Shaded columns: drug followed by BAL-treatment.

Results. Ten experiments were performed with tartar emetic administered either intraperitoneally or intramuscularly. In every experiment except one, treatment with BAL either increased the survival period or prevented death, in comparison with groups of rats simultaneously receiving only the solution of tartar emetic. Illustrative experiments are shown in Fig. 1 and 2. A control experiment with very large doses of HgCl_2 as the lethal agent is shown in Fig. 3; an expected protective action of BAL was demonstrated although more survivals after the last dose probably would have been observed had BAL treatment been continued after the last injection of HgCl_2 solution. In the one inconsistent experiment, tartar emetic in increasing doses was injected once daily intraperitoneally into each rat of 3 groups of 10 each. The initial dose was 30 mg per kg and the last dose, on the 11th day, was 60 mg per kg. However, only 20% of the control rats died whereas of those receiving BAL in oil in addition to tartar emetic, 80% of the second group (20 mg of BAL per kg immediately after tartar emetic) and 30% of the third group (20 mg of BAL per kg im-

mediately after tartar emetic and repeated 3 hours later) did not survive. The subcutaneous tissues of the animals treated with BAL contained several milliliters of oil at the end of the experiment. No satisfactory explanation of the deleterious effect of BAL-treatment or of the remarkable tolerance of the control rats to intraperitoneally-administered tartar emetic in this experiment has been found.

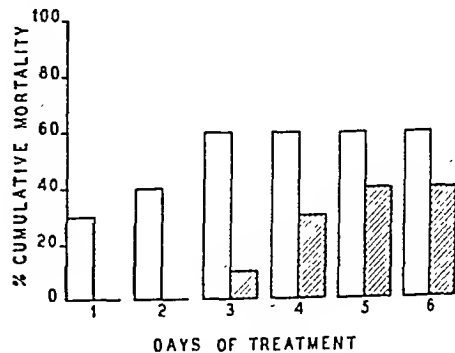


FIG. 2.

Effect of BAL in oil subcutaneously on survival after intraperitoneal injection once daily of 25 mg of tartar emetic per kg in aqueous solution. One dose of BAL (20 mg per kg) was given immediately after each of the 6 injections of tartar emetic. There were no deaths after the 6th injection. Each group consisted of 10 rats.

White Columns: drug alone.

Shaded columns: drug followed by BAL-treatment.

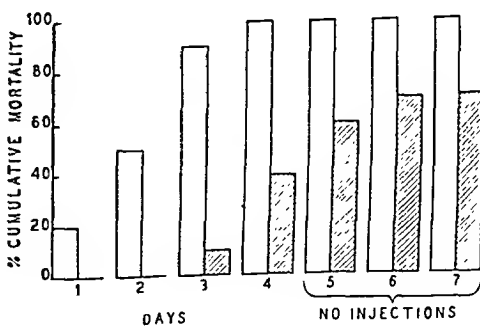


FIG. 3.

Effect of BAL in oil subcutaneously on survival after intraperitoneal injection of 5 mg of HgCl_2 per kg once daily at beginning of each of first 4 days. One dose of BAL (20 mg per kg) was given immediately after each injection of HgCl_2 solution. The 3 surviving rats were sacrificed. Each group consisted of 10 rats.

White columns: HgCl_2 alone.

Shaded columns: HgCl_2 and BAL.

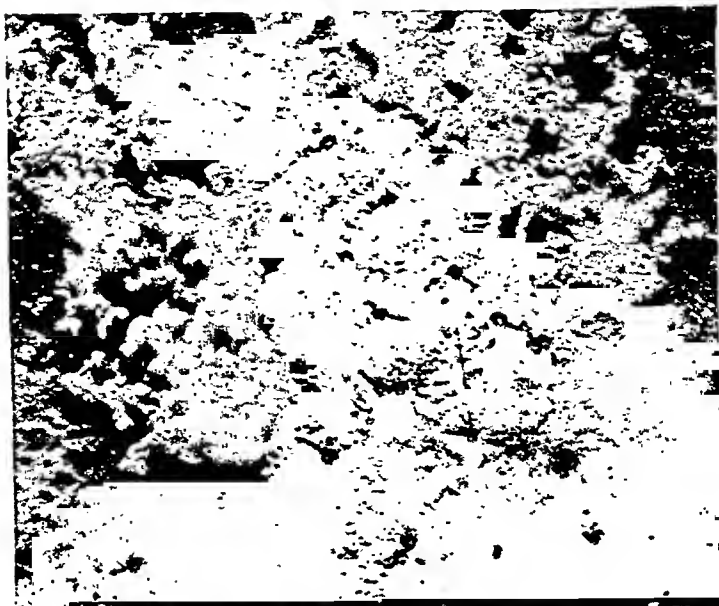


FIG. 1.

An electron micrograph of a gold-shadowed collodion replica of the surface of an *E. coli*-bacteriophage culture. The clear area of the plaque, besides showing a few adhering bacteriophage particles, is covered with pits which presumably are replicas of the heads of other particles. The finer granulation of this and the succeeding photographs is the macromolecular texture of collodion as brought out by the shadowing. Magnification *ca.* 19000 \times .



FIG. 2.

A clump of bacteriophage particles just inside the edge of a plaque area on a Petri dish culture. Magnification *ca.* 24,000 \times .

aqueous solutions of drug or of drug and of BAL. The lethal effects of the other 2 antimonials used by these authors, Fuadin and Neostam, contrary to their results, were found clearly to be enhanced by the administration of BAL. Similarly, BAL increased the lethal action of poisonous doses of Neostibosan. The dimercaptan did not alter the death rate from large intraperitoneal doses of Stibanose. The doses of BAL, whether dissolved in peanut oil or in water, were sufficient to save the lives of animals receiving lethal doses of either mercuric chloride or sodium arsenite. BAL in oil was administered in doses well below levels which by themselves are followed by any manifestation of poisoning. The largest intraperitoneal dose of aqueous solution of BAL used (about 60 mg per kg) can cause central stimulation in some rats, as shown by generalized muscular hyperactivity; however, this dose, followed by 30 mg per kg 4 hours later, can be given daily for at least 3 days without killing any animals.

Other experiments will have to be performed to determine, if possible, what is the explanation of the increased toxicity of

Fuadin, Neostam and Neostibosan, if BAL be administered a short time later. The increased mortality associated with BAL-treatment occurred mainly within 24 hours; a significant difference in mortality was evident after 6 hours. It is possible that an acutely toxic complex substance is formed *in vivo* by the interaction of the dimercaptan and any of the 3 antimonials which themselves are not well-defined compounds; however, it is more acceptable to interpret the increased mortality as a summation of toxic effects. It seems reasonable to conclude that the favorable action of BAL in rats acutely poisoned by tartar emetic is the result of the formation of one or more mercaptides of antimony much less toxic than tartar emetic, just as is the case when an arsenical or a mercuric salt is rendered innocuous by the administration of BAL.

Summary. In rats, the acute lethal action of tartar emetic is significantly reduced by the administration of BAL. BAL increases the mortality rate in rats receiving Fuadin, Neostam or Neostibosan. The mortality rate in rats given large doses of Stibanose is not reduced by BAL.

15684 P

Electron Micrographs of Bacterial Cultures Infected with Bacteriophage.

O. F. EDWARDS AND RALPH W. G. WYCKOFF.

From the National Institute of Health, Bethesda, Md.

Now that we have learned to recognize individual virus particles through the electron microscopy of purified suspensions it has become practical to approach the more important question of how these particles are produced within the cells they infect. Probably bacteria with their bacteriophage provide the simplest virus system immediately accessible to existing technics. This is partly because sperm-like bacteriophage particles^{1,2} can easily be recognized even in the presence of other particles of similar sub-

microscopic size and partly because bacteria are hosts small enough to allow an electron microscopic examination of both their surface and their internal structures.

To gain a satisfactory understanding of the steps involved in bacteriophage-production it is essential to be able to photograph sensitive bacteria under the conditions in which they grow and become infected. We have found that this can be done by studying replicas made of the surfaces of agar

¹ Luria, S. E., Delbrück, M., and Anderson, T. F., *J. Bact.*, 1943, **40**, 57.

² Sharp, D. G., Taylor, A. R., Hook, A. E., and Beard, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61**, 259.

Typical micrographs of replicas made in the way just described are shown in Fig. 1-3. They are from the periphery of plaques developed on Petri dishes inoculated with a mixture of *E. coli* and a T2 strain of its bacteriophage. A number of isolated bacteriophage particles adhering to the film are evident in the clear areas of Fig. 1; the film also shows numerous pits that are undoubtedly replicas of particles that remained on the agar. The mass at the left of this micrograph, which approximately outlines the remains of a single bacterial cell, is a typical example of how completely bacterial proto-

plasm becomes converted into bacteriophage particles after invasion by one of them. Similar masses within the confines of old cells are reproduced at higher magnifications in Fig. 2 and 3. The separate particles can readily be recognized even when, as is commonly the case, they have partially collapsed presumably as a consequence of drying. These pictures do not reveal in detail how the bacteriophage particles are formed but they do suggest that a careful study of films taken from cultures containing bacteria in various stages of infection may be expected to yield such information.

15685

Sugar Alcohols. XXV. Sorbitol and Sorbitan as Precursors of Liver-Glycogen in the Rat.*

C. JELLEFF CARR, DE CÂMP. B. FARSON, AND JOHN C. KRANTZ, JR.

From the University of Maryland, Baltimore, Md.

For many years we have been concerned with the fate of the sugar alcohols in the animal body.¹ Previously it was shown that mannitol served as a precursor of glycogen in the liver of the rat, whereas its anhydrides mannitan and isomannide were not available in this capacity. Sorbitol, an isomer of mannitol, surpasses the latter as a glycogen former. Having available crystalline sorbitan, anhydrosorbitol, it occurred to us to study its availability as a source of liver glycogen. Previously Carr and Krantz showed that polygalitol-1,5-anhydro-D-sorbitol was not available as a precursor of glycogen in the rat.² The relation of sorbitol to sorbitan is shown by the following formulas.

Experimental. Young white male rats from a uniform source, weighing from 150 to 200 g

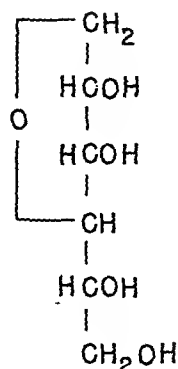
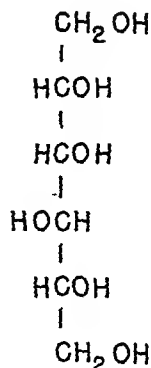
* The sorbitol and sorbitan were generously supplied by the Atlas Powder Company of Wilmington, Del.

¹ Carr, C. J., and Krantz, J. C., Jr., *Advances in Carbohydrate Chemistry*, Vol. I, p. 175-192.

² Carr, C. J., and Krantz, J. C., Jr., *J. Biol. Chem.*, 1934, 107, 371.

were fed a balanced ration (Purina Chow) for 3 days. They were then fasted for 48 hours and divided into the following experimental groups according to diet.

1. Cacao butter controls.
2. Cacao butter plus 30% crystalline sorbitol.



D-SORBITOL
M.p. 97° [α]_D -1.97°

D-SORBITAN
Probable structure
M.p. 113°

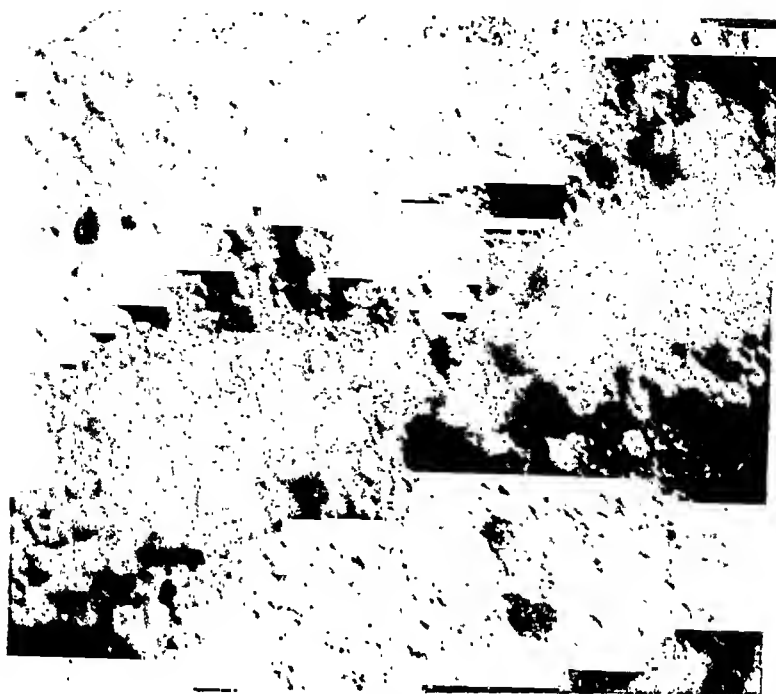


FIG. 3.
Clumps of bacteriophage filling the approximate outlines of two recently divided bacteria. Magnification ca. 35,000 \times .

plates incubated for various periods of time after inoculation with bacteria and bacteriophage. The technic we have been using to obtain such replicas is essentially the same as that recently described by Hillier and Baker.³ It has consisted in covering the surface of a bacteriophage-bacterial growth with a dilute solution of collodion or other plastic and floating the resulting film off onto a water surface for further manipulation. Like Hillier and Baker we have found that organisms are retained by the film made in this way but our preparations have also shown numerous impressions of individual bacteria. This has made it possible to see in a single film details of both surface and internal structure.

Formvar dissolved in ethylene dichloride will yield good replicas but best results have been obtained with collodion. Collodion, USP, diluted with 5 to 6 volumes of normal amyl acetate, carefully spread over the

growth-surface and drained till dry has produced films of the proper thickness. The degree of moistness of the surface has had a dominant influence on both the quality and the usefulness of the plastic film. When it was too wet the replica has reproduced little besides micro-drops of water covering its surface; when it was too dry masses of bacteria too thick for observation adhered to the finished replica. Contrast for microscopy has been introduced into the replicas floated from these Petri dishes by shadowing⁴ them obliquely with metal to a calculated thickness of ca 8A of gold or ca 40A of chromium. Such shadowed replicas have been so thin that they have often stretched and distorted under the electron beam especially in and about regions of bacterial replication. This could be minimized by lightly metallizing the finished shadowed replicas through the vertical deposition of a few angstroms' thickness of aluminum, chromium or beryllium.

³ Hillier, J., and Baker, R. F., *J. Bact.*, 1946, 52, 411.

⁴ Williams, R. C., and Wyckoff, R. W. G., *J. Applied Physics*, 1946, 17, 23.

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15685

Sugar Alcohols. XXV. Sorbitol and Sorbitan as Precursors of Liver-Glycogen in the Rat.*

C. JELLEFF CARR, DE CAMP. B. FARSON, AND JOHN C. KRANTZ, JR.

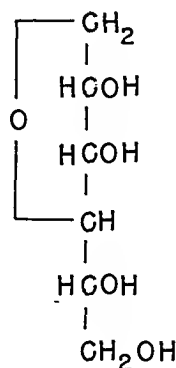
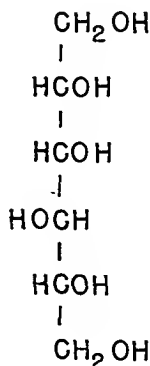
From the University of Maryland, Baltimore, Md.

For many years we have been concerned with the fate of the sugar alcohols in the animal body.¹ Previously it was shown that mannitol served as a precursor of glycogen in the liver of the rat, whereas its anhydrides mannitan and isomannide were not available in this capacity. Sorbitol, an isomer of mannitol, surpasses the latter as a glycogen former. Having available crystalline sorbitan, anhydrosorbitol, it occurred to us to study its availability as a source of liver glycogen. Previously Carr and Krantz showed that polygalitol-1,5-anhydro-D-sorbitol was not available as a precursor of glycogen in the rat.² The relation of sorbitol to sorbitan is shown by the following formulas.

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were fed a balanced ration (Purina Chow) for 3 days. They were then fasted for 48 hours and divided into the following experimental groups according to diet.

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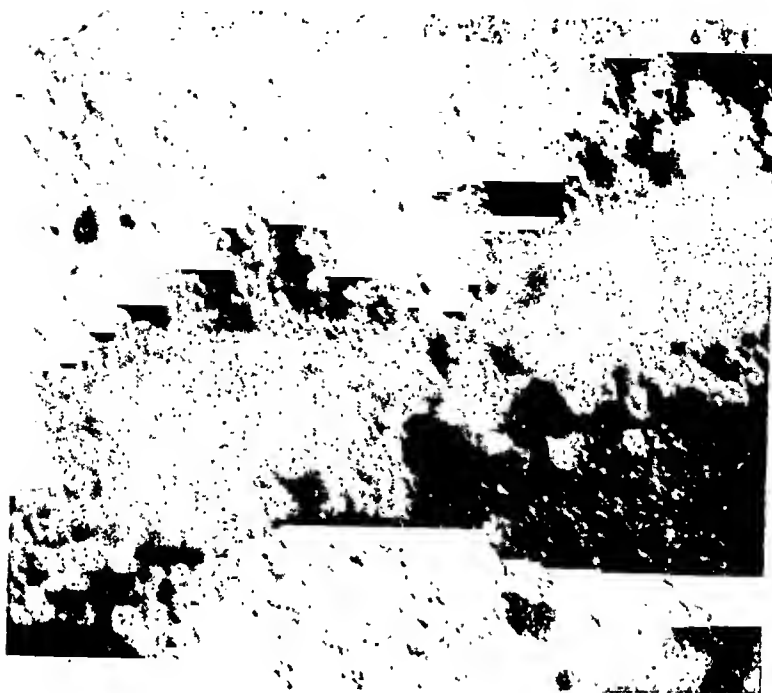


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TABLE I.
Average Concentration* of Streptomycin (Units/g of Tissue) in Blood, Liver, and Spleen of Mice.

Hr after injection	Concentration of streptomycin in animals which received					
	Streptomycin-Trypan Blue†			Streptomycin‡		
	Blood	Liver	Spleen	Blood	Liver	Spleen
1	12.0	12.8	59.4	4.0	5.1	0
4	2.5	2.8	6.1	0	0	0
8	0.9	4.2	4.0	0	0	0
25	0.6	6.0	4.9	0	0	0

* The micro techniques of streptomycin assay of Forgaes and Kucera (*J. Lab. and Clin. Med.*, in press) were used in this experiment.

† Each of the 2 large groups contained 20 animals: 5 animals in each group were sacrificed for examination at the post-injection intervals shown.

the inability of the antibiotic to reach intracellularly contained etiologic agents.² To test this supposition, intracellular introduction of streptomycin into the reticulo-endothelial cells was attempted by combining the antibiotic with an electronegatively-charged colloid that could be phagocytized. Preliminary experiments consisted of injecting white Swiss mice intraperitoneally with a single dose of a mixture containing 1000 "S" units of streptomycin and 20 mg of an electronegatively-charged dye, trypan blue, in 1 ml of water. The mixture was adjusted to pH 7.4. (In contrast to streptomycin *per se*, this mixture of antibiotic and colloid did not dialyze through a cellophane membrane over a period of 24 hours).³ Three control groups of animals received respectively (a) 1000 "S" units of the antibiotic dissolved in 1 ml of distilled water, (b) 20 mg of the colloid suspended in 1 ml distilled water, and (c) 1 ml of distilled water. Blood and organs rich in reticulo-endothelial tissue were assayed for the presence of the antibiotic one, 4, 8, and 25 hours after the injection. The average results are presented in Table I.

It will be observed that the group receiving streptomycin in distilled water showed levels of the antibiotic in the blood and liver

only in the one-hour samples. The group receiving the antibiotic and dye mixture showed relatively high levels of streptomycin in blood, liver and spleen at all intervals of testing from one through 25 hours. In every case the unitage per gram of liver or spleen in this group always exceeded that of the comparable blood sample. Since the other 2 control groups remained negative throughout the experiment they are not included in Table I. Because of the substantially higher unitages in the liver and spleen of the group which received the antibiotic and dye as opposed to levels in samples of blood taken at comparable times, it was considered reasonable to assume from these data that the antibiotic was concentrated in these organs. Furthermore, histological examination of the sections showed a characteristic distribution of the colloidal dye in the reticulo-endothelium. Results obtained using guinea pigs confirmed the phenomenon observed in mice.

From the above data, it was considered that such a method of altering the distribution of therapeutic substances and delaying their excretion, might be developed for treatment of certain diseases caused by obligately or facultatively-intracellular parasites.⁴ To this end, further experiments are being conducted and will be reported at a later date.

² Goodpasture, E. W., *Trans. and Studies of the College of Physicians of Philadelphia*, 1941, 9, 11.

³ Grateful acknowledgment is made to Dr. Walter Koeholaty for his suggestions and help concerning this phase of the work.

The authors wish to acknowledge their gratitude to Margery T. Fell, Lois L. Chaney, and James W. Brown for their technical assistance, and to Dr. William J. Cromartie for his suggestions and advice.

TABLE I.
Glycogen Storage in Liver of White Rat After Feeding Sorbitol and Sorbitan in a Cacao Butter Diet.

Cacao butter		Sorbitol		Sorbitan	
No. of rats	Liver glycogen, %	No. of rats	Liver glycogen, %	No. of rats	Liver glycogen, %
3	Less than 0.1	3	0.85	3	0.05
3		8	1.16	3	0.02
3		5	1.37	3	0.04
3				3	0.04
3				3	0.05
				3	<0.01
				3	<0.01

3. Cacao butter plus 30% crystalline sorbitan.

Several animals in each group were divided into separate cages containing 3 to 8 rats each. The animals were fed their respective diets for 2 days. At the end of the feeding period the animals were anesthetized with intraperitoneal injections of amytal sodium and their livers extirpated. The livers in each group were pooled. The glycogen

was determined by the method of Good *et al.*,³ and the dextrose obtained by its hydrolysis was determined by the Munson-Walker method. The results are shown in Table I.

Conclusion. The data in Table I show that sorbitol and sorbitan behave like their isomers mannitol and mannitan⁴ with respect to glycogen storage in the liver of the white rat. Mannitol and sorbitol are precursors of glycogen whereas their anhydrides are not.

³ Good, C. A., *et al.*, *J. Biol. Chem.*, 1933, 100, 485.

⁴ Carr, C. J., *et al.*, *J. Biol. Chem.*, 1933, 102, 721.

15686 P

Alteration of the Distribution and Excretion of Streptomycin.*

WILLIAM E. NELSON, JOSEPH FORGACS, AND JOSEPH L. KUCERA.
(Introduced by Edwin H. Lennette.)

From Camp Detrick, Frederick, Md.

Phagocytosis of colloidal particles by the reticulo-endothelium and the localization of such particles in inflamed areas under certain conditions are well known phenomena. These processes apparently depend upon several factors, among which are (a) charge of the particle, (b) particle size, and (c) capillary permeability.

It has been observed that penicillin¹ and streptomycin,² while effective in eliminating

the septicemic phase of certain diseases, fail to provide a complete cure. In the light of previously reported evidence,³ indicating that liver and spleen levels of streptomycin remain negative while blood levels are within a therapeutic range, it might reasonably be supposed that therapeutic failure⁴ is due to

* Streptomycin used in these experiments was streptomycin hydrochloride.

¹ Robinson, H. J., Thesis, Rutgers University, New Brunswick, N.J., 1943, 43.

² Keefer, C. S., Blake, F. G., Lockwood, J. S., Long, P. H., Marshall, E. K., and Wood, B., Jr., *J. A. M. A.*, 1946, 132, 9.

³ Kornegay, G. B., Forgacs, J., and Henley, T. F., *J. Lab. and Clin. Med.*, 1946, 31, 523.

⁴ Kelly, E. G., and Henley, T. F., personal communication.

TABLE I.

Composite Table of Excretion of Streptomycin in Man, Dog, and Monkey as Reported in the Literature.

Animal	Streptomycin administered		% recovered in urine in 24 hr or longer	Reference
	Route	Dose \times 1000 (units)		
Man	I.M.	25	6.1-6.5	(8)
	"	50	19.5	(8)
	"	100	59.1-68.1	(4)
	"	200	42-78.2	(8)
	"	200	63 (4 hr)	(6)
	"	300	72	(6)
	"	400	16.7-22.2	(4)
	"	400	41-53.8	(8)
	"	500	56-71	(8)
	"	600	45.5-75.5	(5)
	"	600	87 (12 hr)	(6)
	"	20,000 in 6 days	44	(3)
	"	varied, over 29 days	30†	(2)*
	"	varied, over 25 days	103‡	(2)*
	I.V.	50	50-85.6	(8)
	"	100	52.3-80.0	(4)
	"	200	64-91.7	(8)
	"	400	62.9	(4)
	"	400	42.7-48	(8)
	"	500	79-83.5	(8)
	"	600	29-89§	(7)
	"	28,000 in 7 days	70	(3)
Monkey	I.M.	10 per kg/day	60-66	(9)
	"	50 per kg/day	38-44	(9)
Dog	I.M.	Not indicated	ca. 50-80	(9)

- Calculated from the published data.

† Total recovery over 29-day period of treatment.

‡ Total recovery over 25-day period of treatment.

§ Average recovery of streptomycin in the urine in 10 cases was 66%.

|| Recoveries published as approximate figures.

Absorption and Excretion of Streptomycin in Mice. Procedure. Large groups (usually 100) of female Swiss mice weighing between 19 and 21 g were given a single dose of streptomycin subcutaneously or intramuscularly. At given intervals, beginning 15 to 30 minutes after injection, 10 mice were sacrificed by bleeding from the heart, the blood pooled, and assays run on the serum. Immediately prior to each bleeding period, urine samples were taken from all surviving mice (by applying slight pressure over the bladder region) and the urine pooled, measured and assayed by the broth dilution method.^{10*}

¹⁰ Donovan, R., Hamre, D., Kavanagh, F., and Rake, G., *J. Bact.*, 1945, 50, 623.

* The various sera studied caused no apparent inhibition of the test organisms. However, assays of sera to which small amounts of streptomycin were added gave results ranging from 34-40% higher than aqueous controls. The streptomycin concentrations were such in these assays that they were carried out on undiluted or low dilutions of serum. Similar studies with low dilutions of urine

Results. In Fig. 1 are shown the serum levels reached in mice following a single injection of streptomycin at 3 dose levels. Also included, for purposes of comparison, are a composite curve of similar data we have obtained in infants as well as a curve showing data taken from the work of Buggs, *et al.*⁸ In the latter case, the body weight of the patient was not indicated and hence the dosage cannot be given in terms of units per kg. It will be noted that serum levels of streptomycin in mice drop considerably faster than in humans following a single dose of the antibiotic.

The excretion rates as shown by the appearance of antibiotic in the urine of mice as compared to data in humans reported by

gave results of 9-23% higher than controls. However, in the absorption-excretion studies in mice the streptomycin concentrations in most of the serum and urine samples were great enough to require considerable dilution of the sample for assay, thereby minimizing this effect to an extent that it may be neglected in the calculations.

The Use of the Mouse in Studies on Streptomycin.

GEOFFREY RAKE AND RICHARD DONOVICK.

From the Division of Microbiology, The Squibb Institute for Medical Research, New Brunswick, N.J.

The therapeutic value of a drug is determined by 2 general aspects; namely, (a) its physiologic distribution in the host and (b) its specific biologic activity. The physiologic distribution may vary greatly with the host as has been pointed out by Marshall¹ with respect to the sulfonamides. The situation with regard to streptomycin is not clear because of widely divergent results which have been published by various investigators.

The mouse, being extensively used in chemotherapeutic studies, is of particular importance in the study of antibiotic substances where the quantities of drug available for research may be sharply limited. Consequently, knowledge of the comparative behavior of streptomycin in mouse and man is of considerable value. The wide range of results which have been reported in the numerous absorption-excretion studies performed in man is summarized in Table I. Aside from an expected variation in individuals, and probably in the assay method and technics, the explanation for the divergence is not clear.

Absorption of streptomycin in mice, as indicated by blood level studies, has been reported^{2,3} but no information has been made

available on excretion rates. Such data are essential if one is to examine whether streptomycin is destroyed in the mouse to a greater extent than in man.² During the past year and a half we have carried out a number of absorption-excretion studies of streptomycin in the mouse as well as in humans (adults and children). Since our findings have bearing on the question of the relative rates of excretion of this antibiotic in these 2 species it was thought to be of interest to present some of these results.

ABSORPTION OF STREPTOMYCIN IN MICE

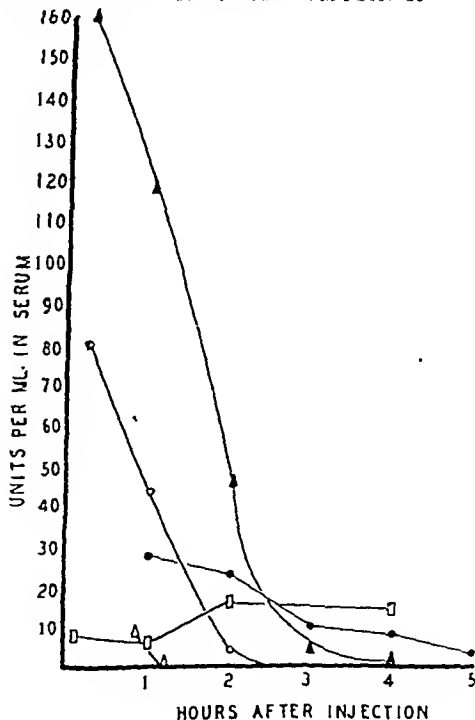


Fig. 1.

▲ 250,000 units per kg, subcutaneously.

○ 104,000 " " " " " "

△ 23,000 " " " " " intramuscularly.

● 1,140 " " " " " " " in

children.

□ 500,000 units, intramuscularly in man, cf Buggs, *et al.*⁸

¹ Marshall, E. K., Jr., *University of Pennsylvania Bicentennial Conference, Chemotherapy*, University of Pennsylvania Press, 1941.

² Kornegay, G. B., Forgaes, J., and Henley, T. F., *J. Lab. and Clin. Med.*, 1946, **31**, 523.

³ Elias, W. F., and Durso, J., *Science*, 1945, **101**, 589.

⁴ Heilman, D. H., Heilman, F. R., Hinshaw, H. C., Nichols, D. R., and Herrell, W. E., *Am. J. Med. Sci.*, 1945, **210**, 576.

⁵ Rutstein, D. D., Stebbins, R. B., Cathcart, R. T., and Harvey, R. M., *J. Clin. Invest.*, 1945, **24**, 898.

⁶ Anderson, D. G., and Jewell, M., *New England J. Med.*, 1946, **233**, 485.

⁷ Zintel, H. A., Flippin, H. F., Nichols, A. C., Whiley, M. M., and Rhoads, J. E., *Am. J. Med. Sci.*, 1945, **210**, 421.

⁸ Buggs, C. W., Pilling, M. A., Bronstein, B., and Hirshfeld, J. W., *J. Clin. Invest.*, 1946, **25**, 94.

⁹ Stebbins, R. B., Graessle, O. E., and Robinson, H. J., *Proc. Soc. Exp. Biol. and Med.*, 1945, **60**, 68.

It was found that the excretion rate of the streptomycin administered together with alum was similar to that of streptomycin alone. The calculated recovery of the antibiotic in the urine was somewhat higher but not significantly so. Apparently the alum did not retard excretion. However, in all previous excretion studies in mice, the urine collected during the first hour or 2 following injection was a deep, reddish brown in color, very similar to that of the concentrated streptomycin solutions used. In the alum study, on the other hand, the urine was normal in color throughout the experiment, indicating that some of the pigmented impurities of the streptomycin preparation had been retained by the alum while the streptomycin was not.

Excretion of Streptomycin in Humans. Considerable data on the absorption and excretion of streptomycin in humans has already been reported (Table I). Consequently no special attempt was made to collect extensive data on this species. Limited data on streptomycin excretion have been collected on one adult and 3 infants to compare recovery rates in the urine with those found in mice, using the same bio-assay procedure.¹⁰

An adult patient, weighing 160 lb. was given 1,850,000 units of streptomycin subcutaneously in divided doses over a 6-day period. The average daily dose was 4,200

units per kg. The overall recovery of antibiotic in the urine was 75% of the streptomycin administered, but the ratio of units administered to units recovered in the urine varied somewhat during various stages of the study.

In 3 small children receiving 20,000 units of streptomycin per lb (ca 9100 units per kg) per day, intramuscularly, in interrupted doses or by continuous drip, the streptomycin administered which could be accounted for in the urine at any given period during the course of treatment again varied considerably with the individual. The overall recoveries of antibiotic in the urine were 31.5, 45.0 and 45.5% respectively. Although these are lower recoveries than some reported in adults, they fall within the general range found in man.

Conclusions. Whereas streptomycin is absorbed and excreted considerably faster in mice than in man, the percentage recovery of streptomycin in the urine of these 2 species is very similar. Hence, there is no reason to believe that this antibiotic is destroyed in mouse to any greater extent than in man.

Despite the fact that streptomycin is adsorbed on alkaline alumina, streptomycin administered in mixture with alkaline potassium aluminum sulfate is excreted as rapidly in mice as is streptomycin given alone.

15688 P

Proteins with Growth-Promoting Action on Tissue Cells *in vitro*.

CHARITY WAYMOUTH. (Introduced by A. Fischer.)

From Carlsberg Fund Biological Institute, Copenhagen, Denmark.

Among the many substances which contribute to the effectiveness of embryonic tissue extracts as growth-promoting media for cells *in vitro*, some have been found to be associated with the proteins of the extracts. The ease of inactivation of the extracts by heat and by proteolytic enzymes were early indications that proteins played some role in the effects which result in increasing the area of explanted tissue relative to the area of sister control-cultures.

The lability of the high molecular sub-

stances in the embryonic extracts makes it necessary to use only very mild methods in attempts to extract or purify active fractions. It has been found by Fischer and Astrup¹ that a rather high activity was associated with a fraction separated by the method used by Hammarsten² for preparation of nucleoproteins. Experiments have now confirmed

¹ Fischer, A., and Astrup, T., *Pflüg. Arch. ges. Physiol.*, 1943, **247**, 34.

² Hammarsten, E., *Hoppe-Seyl Z.*, 1920, **100**, 141.

It was found that the excretion rate of the streptomycin administered together with alum was similar to that of streptomycin alone. The calculated recovery of the antibiotic in the urine was somewhat higher but not significantly so. Apparently the alum did not retard excretion. However, in all previous excretion studies in mice, the urine collected during the first hour or 2 following injection was a deep, reddish brown in color, very similar to that of the concentrated streptomycin solutions used. In the alum study, on the other hand, the urine was normal in color throughout the experiment, indicating that some of the pigmented impurities of the streptomycin preparation had been retained by the alum while the streptomycin was not.

Excretion of Streptomycin in Humans. Considerable data on the absorption and excretion of streptomycin in humans has already been reported (Table I). Consequently no special attempt was made to collect extensive data on this species. Limited data on streptomycin excretion have been collected on one adult and 3 infants to compare recovery rates in the urine with those found in mice, using the same bio-assay procedure.¹⁰

An adult patient, weighing 160 lb, was given 1,850,000 units of streptomycin subcutaneously in divided doses over a 6-day period. The average daily dose was 4,200

units per kg. The overall recovery of antibiotic in the urine was 75% of the streptomycin administered, but the ratio of units administered to units recovered in the urine varied somewhat during various stages of the study.

In 3 small children receiving 20,000 units of streptomycin per lb (ca 9100 units per kg) per day, intramuscularly, in interrupted doses or by continuous drip, the streptomycin administered which could be accounted for in the urine at any given period during the course of treatment again varied considerably with the individual. The overall recoveries of antibiotic in the urine were 31.5, 45.0 and 45.5% respectively. Although these are lower recoveries than some reported in adults, they fall within the general range found in man.

Conclusions. Whereas streptomycin is absorbed and excreted considerably faster in mice than in man, the percentage recovery of streptomycin in the urine of these 2 species is very similar. Hence, there is no reason to believe that this antibiotic is destroyed in mouse to any greater extent than in man.

Despite the fact that streptomycin is adsorbed on alkaline alumina, streptomycin administered in mixture with alkaline potassium aluminum sulfate is excreted as rapidly in mice as is streptomycin given alone.

15688 P

Proteins with Growth-Promoting Action on Tissue Cells *in vitro*.

CHARITY WAYMOUTH. (Introduced by A. Fischer.)

From Carlsberg Fund Biological Institute, Copenhagen, Denmark.

Among the many substances which contribute to the effectiveness of embryonic tissue extracts as growth-promoting media for cells *in vitro*, some have been found to be associated with the proteins of the extracts. The ease of inactivation of the extracts by heat and by proteolytic enzymes were early indications that proteins played some role in the effects which result in increasing the area of explanted tissue relative to the area of sister control-cultures.

The lability of the high molecular sub-

stances in the embryonic extracts makes it necessary to use only very mild methods in attempts to extract or purify active fractions. It has been found by Fischer and Astrup¹ that a rather high activity was associated with a fraction separated by the method used by Hammarsten² for preparation of nucleoproteins. Experiments have now confirmed

¹ Fischer, A., and Astrup, T., *Pflüg. Arch. ges. Physiol.*, 1943, **247**, 34.

² Hammarsten, E., *Hoppe-Seyl Z.*, 1920, **109**, 141.

that such fractions contain the relevant activity. It is, however, not yet certain that the activity necessarily resides in a protein, as distinct from a protein-complex containing nonprotein material, for one of the results of using mild preparative procedures is that labile, loose linkages may not be broken.

Examination of some crude nucleoprotein fractions has revealed no correlation between the activity and the proportions of the 2 types of nucleic acid, *i.e.* those containing ribose and desoxyribose. Activity has been found in fractions containing only a trace of desoxyribonucleic acid, so it is concluded that, if a nucleic acid is essential for activity, it must be one of the ribose and not of the desoxyribose type. Ribonucleic acid alone, in the depolymerised form in which it is isolated, does not appear to possess the activity. No method has so far been found for dissociating the ribonucleic acid from the protein, with which it is in firm combination, without denaturing the protein, so it cannot be stated whether the protein moiety alone shows any effect.

A convenient material for studying the active protein fraction of embryonic extracts has been prepared by precipitation of extracts in the cold (-15°C) by a modification of the method used by Hardy and Gardiner³ and others for delipidation of serum and plasma proteins. Embryonic extracts are made by mixing equal parts of tissue pulp and Ringer solution, and they therefore contain only material relatively easily extractable from the tissue. Such extracts, and hence also the powders prepared from them by cold-precipitation, are almost completely free from desoxyribonucleic acid. The stable, dry preparations of tissue protein obtained in this way give solutions in saline which possess the activity under discussion.

Examination of solutions of some of the powders so prepared in the Tiselius electrophoresis apparatus revealed that chick embryo preparations contain components of relatively few different mobilities. Preparations from calf embryos were even simpler

in this respect, showing, in buffers of 2 different pH values, only a narrow range of mobilities. It was therefore thought to be of interest to examine the sedimentation of such a preparation in the ultracentrifuge; and through the courtesy of Dr. K. O. Pedersen of Upsala, this was done. For comparison with the calf embryo preparation, a powder prepared in exactly the same manner from the muscle of adult cow was also examined. The cow muscle preparation contained, as would be expected, several components, notably those with sedimentation constants (S_{20}) in the region of 1, 5 and 8. Electrophoretically this preparation was relatively homogeneous. The embryonic calf preparation, on the other hand, contained only one principal component, with $S_{20} = 4.8$, and corresponding therefore in size with the albumin group of proteins.

Of the low molecular substances precipitated with the proteins by the Hardy and Gardiner technic, a special examination of the phosphorus fractions has been made, since, as in plasma (Waymouth and Davidson⁴), a large proportion of inorganic and acid-soluble phosphorus appears in the final product. The greater part of these fractions could, however, be removed from the extract before precipitation, by dialysis overnight against running tapwater at about 10°C , without affecting the activity of the protein powder upon the growth of the tissue cultures.

These preliminary experiments have demonstrated that a type of growth-stimulating activity is associated with a protein fraction containing ribonucleoprotein, and possessing, for a material prepared by such a crude procedure, a surprisingly high degree of homogeneity in electrophoretic migration and in ultracentrifugal sedimentation. Growth-promoting activity was also found in adult tissue preparations made in a similar way, and it is thought that the type of substance responsible for the effect may be widely distributed in tissue cells, though masked in adult tissue extracts by the relatively higher proportion of nonactive components.

³ Hardy, W. B., and Gardiner, S., *J. Physiol.*, 1910, **40**, lxviii.

⁴ Waymouth, C., and Davidson, J. N., *Biochem. J.*, 1946, **40**, proc.

Changes in Concentration of Enzymes in Pancreatic Juice After Giving Insulin.*

J. E. THOMAS AND J. O. CRIDER.

From the Department of Physiology, Jefferson Medical College.

Babkin¹ has recently reviewed the literature dealing with the effects of hypoglycemia and hyperglycemia on the external secretory function of the pancreas, hence only a brief summary of the conclusions to be drawn from previous experimental work will be given here. In cross-circulated dogs hypoglycemia of the head diminishes and hyperglycemia augments the output of fluid and enzymes from the pancreas (La Barre and Destrée²⁻⁴). In rabbits hypoglycemia decreases the output of enzymes from the pancreas through a central action and hyperglycemia increases the output through a peripheral action (Baxter,⁵ Hebb⁶). Also in rabbits the normal "trophic" effect (increase in enzyme output) of vagus stimulation is reversed by hypoglycemia (Hebb⁶). Experiments on human subjects have given quite different results from those obtained on anesthetized animals. Insulin hypoglycemia stimulates the flow of pancreatic juice in man (Okada⁷) but not if gastric juice is excluded

from the intestines (Frisk and Welin⁸). The same is true of fistula dogs (Scott *et al.*⁹) When given with secretin, insulin increases the output of pancreatic enzymes in man (Lagerlof and Welin¹⁰).

Apparently no information is available regarding the effect of insulin-induced hypoglycemia on the output of pancreatic enzymes in unanesthetized experimental animals. This report is intended as a contribution to the study of that problem.

Methods. The experiments were performed on 3 dogs having tubulated gastric and duodenal fistulas with the duodenal fistula opposite the main pancreatic duct. Pancreatic juice was collected through a temporary glass cannula inserted into the duct via the duodenal fistula.^{9,11} The stomach was drained with the aid of suction through a double drainage tube passed through the gastric fistula all the way to the pylorus. The drainage tube was made up of a rather large soft rubber outer tube within which was a much smaller inner tube. Both tubes had numerous perforations near the pyloric end. The inner tube was connected through a trap to a water-operated vacuum pump.

Because insulin alone does not stimulate pancreatic secretion in the dog,⁹ secretin was given by continuous intravenous injection throughout each experiment by means of a motor-driven pump. The rate of secretin injection was adjusted to give an estimated 3 to 4 cc of pancreatic juice in 10 minutes but these limits were frequently exceeded in practice.

* We are indebted to Dr. M. H. F. Friedman for making the determinations of tryptic activity and to Dr. Friedman and to Wyeth, Inc., for adequate supplies of secretin. We are also indebted to Prof. Abraham Cantarow in whose laboratory the blood sugar determinations were made.

¹ Babkin, B. P., *Secretory Mechanism of the Digestive Glands*, Hoeber, New York, 1942.

² La Barre, J., and Destrée, P., *C. R. Soc. Biol. Paris*, 1928, **98**, 1237; *Ibid.*, 1928, **98**, 1240; *Ibid.*, 1928, **99**, 1056; *Ibid.*, 1928, **99**, 1874; *Ibid.*, 1929, **101**, 147; *Ibid.*, 1935, **110**, 1177.

³ La Barre, J., *C. R. Soc. Biol. Paris*, 1928, **99**, 1053; *Am. J. Physiol.*, 1930, **94**, 117.

⁴ Destrée, P., *C. R. Soc. Biol. Paris*, 1930, **104**, 1038.

⁵ Cited by Babkin.¹

⁶ Baxter, S. G., *Quart. J. Exp. Physiol.*, 1932, **21**, 355.

⁷ Hebb, C. O., *Quart. J. Exp. Physiol.*, 1937, **26**, 339.

⁸ Okada, S., *Nagoya J. med Sci.*, 1933, **7**, 91.

⁹ Frisk, A. R., and Welin, G., *Acta med. scand.*, 1937, **91**, 170.

¹⁰ Scott, V. Brown, Collingnon, U. J., Bugel, H. J., and Johnson, G. C., *Am. J. Physiol.*, 1941, **134**, 208.

¹¹ Lagerlof, H., and Welin, G., *Acta med. scand.*, 1937, **91**, 397.

¹² Hart, Wm. M., and Thomas, J. E., *Gastroenterology*, 1945, **4**, 409.

The dogs were fed once daily and were used for experiments before feeding, when the stomach was generally empty. If any food residues remained they were removed through the fistula before starting the experiment.

Pancreatic juice was collected continuously and divided into separate samples, each collected during a 10-minute interval. After a control period during which at least 5 10-minute samples were collected with secretin alone, insulin was given intravenously in doses ranging from 7.5 to 20 units (total dose). The experiment was then continued for one hour or longer without further modification. Blood samples were collected from a leg vein at intervals throughout each experiment. Blood sugars were determined by the Benedict method.¹²

The volume and specific gravity of each 10-minute sample of pancreatic juice were measured. Specific gravity was determined by weighing the juice in a 1 ml specific gravity bottle at room temperature. The control samples and the samples collected after giving insulin were then poured into separate containers and the proteolytic activity determined on each group of pooled samples. The first sample collected and any additional samples obtained while adjusting the rate of secretin injection were excluded from the control pool.

Results. Sixteen reasonably satisfactory experiments were performed. Four experiments were discarded because of technical difficulties, the most common being the appearance of excessive amounts of acid in the duodenal contents. Only those experiments were discarded in which, in our judgment, the technical failures materially influenced the results.

Blood Sugars. The range of control blood sugar concentrations was approximately the same in the 3 dogs used. The highest control value was 95 mg per 100 ml and the lowest 51 mg. Only 3 were above 70 mg and 3 were below 60 mg. The doses of insulin were sufficient to lower the blood sugar to between 30 and 40 mg in most instances.

In 2 experiments, both in the same animal (2-46) the blood sugar fell to below 30 mg. Restlessness, dilation of the pupils, increase in pulse rate and, occasionally, drowsiness were observed in association with hypoglycemia. Blood sugar values are recorded along with other data in Table I.

Changes in Specific Gravity of Pancreatic Juice. Specific gravity was used as an index of the nitrogen content of the pancreatic juice with which it has a linear relation.¹³ The nitrogen, in turn, is believed to vary with the enzyme content.¹⁴ After giving insulin the specific gravity invariably increased. The increase was generally evident in the first 10-minute sample collected after giving insulin and reached a maximum within 30 to 40 minutes. The subsequent decline was gradual and seldom reached the control level during the one to 2 hours that the observations were continued. Detailed data are given in Table I and a typical experiment is illustrated in Fig. 1. The significance of the changes in specific gravity will be more evident if the estimated values for average total nitrogen before and after giving insulin are compared. Increases of well over 100% of the control value for total nitrogen are common among the average values after insulin. The average values, however, fail to reveal the full effect of insulin since they include data on samples collected before the response was fully developed. In this situation the maximum values are more significant.

Changes in Tryptic Activity. Tryptic activity is expressed in Table I in terms of the velocity constant, k , as determined by the rate of decrease in turbidity of a colloidal suspension of coagulated egg white.^{15,16} It is evident that in the 10 experiments in which enzyme determinations were made the tryptic activity increased with increasing specific gravity in all but 2. In one of these

¹³ Crider, J. O., and Thomas, J. E., *Am. J. Physiol.*, 1944, **141**, 730.

¹⁴ Babkin, B. P., and Tichomirov, N. P., *Z. f. physiol. Chem.*, 1909, **62**, 468.

¹⁵ Friedman, M. H. F., in press.

¹⁶ Riggs, B. C., and Stadie, W. C., *J. Biol. Chem.*, 1943, **150**, 463.

¹² Benedict, S. G., *J. Biol. Chem.*, 1920, **83**, 165; 1928, **70**, 457.

TABLE I.
Effect of Insulin on Pancreatic Secretion Induced by Secretin.

Dog No.	Date 1946	Control period				After giving insulin (first hour)									
		Avg vol. of		Specific gravity, avg	Avg total N (estimated) mg/cc	Trypsin, % [†] mg/cc	Blood sugar, mg/100 cc	Dose of insulin, units	Avg vol. of		Specific gravity		Avg total N (estimated) mg/cc	Trypsin, % [†] mg/cc	Min. blood sugar, mg/100 cc
		10 min., samples, cc	Specific gravity, avg						6 10-min. samples, cc	Max.	Avg				
1-43	5/22	4.5	1.0108	1.96	0.143	77.5	20	3.2	1.0156	1.0131	3.44	0.170*	35		
"	31	4.4	1.0167	1.90		63.7	15	4.31	1.0167	1.0144	4.10		31		
"	6/4	3.9	1.0119	2.61		69	15	5.3	1.0177	1.0158	4.93		33		
"	17	3.9	1.0114	2.32	0.155	68.5	15.1	3.7	1.0154	1.0138	3.75	0.101 [§]	50		
"	7/30	2.8	1.0118	2.55		95.5	15	2.3	1.0184	1.0160	5.05		49		
1-46	6/21	4.8	1.0118	2.55		51.5	20	5.1 [‡]	1.0156	1.0141	3.93		31		
"	7	3.5	1.0109	2.02	0.250	68	10	4.5	1.0145	1.0134	3.51	0.425	36.5		
"	19	5.1	1.0109	2.02	0.110	66	10	3.7	1.0132	1.0121	2.73	0.105	40		
"	24	3.4	1.0092	1.01	0.05	69	7.5	6.3	1.0113	1.0102	1.66	0.09	51		
"	7/1	3.4 [‡]	1.0098	1.36		58	45	4.9	1.0135	1.0119	2.61		34		
2-46	6/5	2.8	1.0104	1.72	0.141	86	20	3.8	1.0192	1.0156	4.81	0.450	22.5		
"	10	4.9	1.0112	2.20	0.260	66	10	3.6	1.0152	1.0126	3.03	0.310	34.5		
"	18	4.2	1.0109	1.48	0.120	52	10	2.6	1.0159	1.0125	2.97	0.19**	28		
"	21	3.0	1.0096	1.25	0.075	60.5	15.11	4.5	1.0116	1.0105	1.78	0.120	55		
"	7/7						5.44	5.4	1.0147	1.0131	3.33	0.260	30		
"	7/8	2.3	1.0111	2.14		68.5	15	2.0	1.0178	1.0159	4.69		36		
"	25	4.4	1.0099 ^{§§}	1.43	0.115 ^{§§}	61.5	7.5	5.4	1.0115	1.0101	1.54	0.170	31		

* 2 samples. Avg sp. gr. 1.0114.

† Traces of acid in duodenum.

‡ Injected subcutaneously.

§ See text.

|| 2 samples omitted because of change in rate of secretin injection.

¶ Acid in duodenum at start, none later.

** 5 samples. Avg sp. gr. 1.0138.

†† Injected subcutaneously.

‡‡ Second dose of insulin; given intravenously.

§§ Includes 4 samples excluded in estimating volume because of difference in rate of secretin injection.

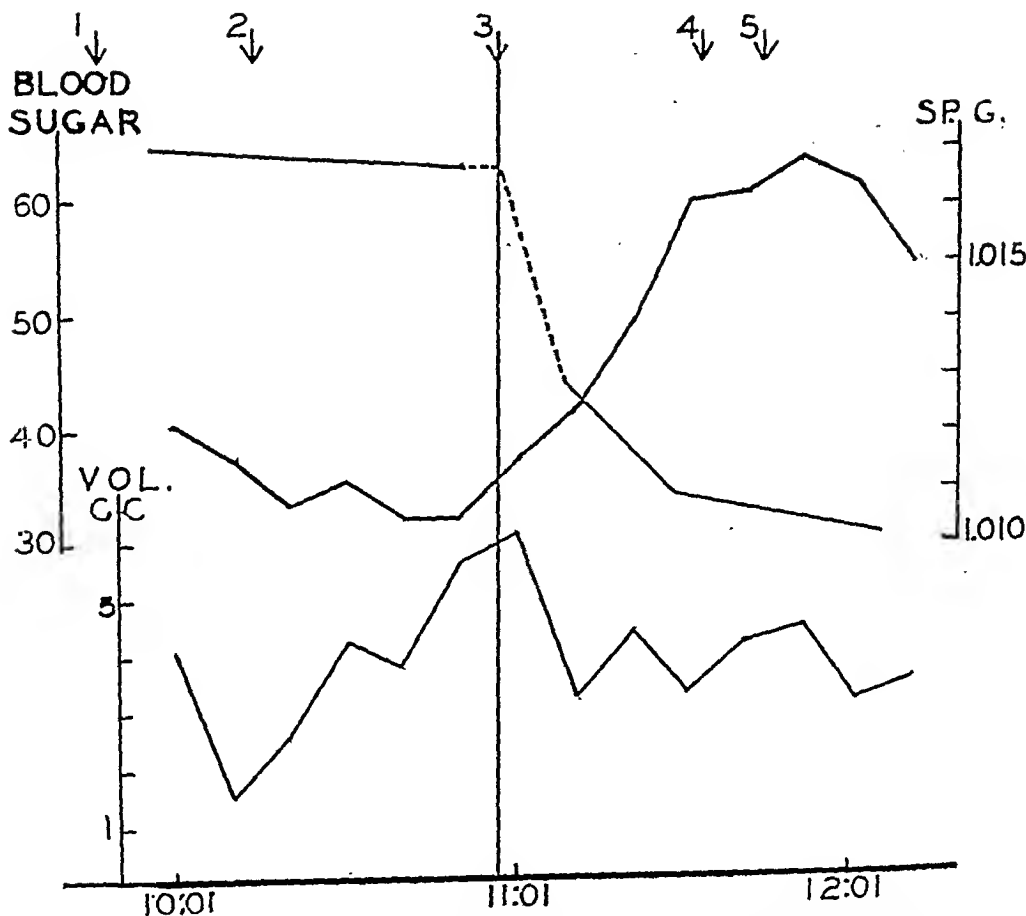


FIG. 1.

Curves showing, in order from above downward, Blood sugar, specific gravity of pancreatic juice, and volume of juice in a typical experiment with insulin on Dog 1-44, May 31, 1946. Meaning of arrows:

1. Secretin injection started.
2. Rate of secretin injection increased from 0.6 mg to 1.0 mg in 10 minutes.
3. Fifteen units of insulin (Mulford) given by vein.
- 4-5. Traces of acid in duodenal contents.

(note "d" in Table I), the experiment was on a dog (1-44) in which discrepancies between tryptic activity, and total nitrogen as determined by the Kjeldahl method, were common. Such discrepancies were rare or did not occur in a considerable number of other dogs that we have studied and we believe this dog to be unique in this respect. In the other instance (Dog 1-46, 6/19/46) the difference is small and probably within the range of experimental error.

Effects on the Volume of Pancreatic Juice.

In 8 of the experiments summarized in Table

I the average volume of the 10-minute samples of pancreatic juice secreted during the first hour after insulin was greater than during the control period. In 5 it was less and in 3 there was practically no change (less than 0.5 cc). The differences in either direction seldom exceed the normal range of variation and are clearly not significant. However, in a few experiments there was a definite decrease in volume after insulin which, when it occurred, became more evident toward the end of the experiment.

1) *Discussion.* According to the available

evidence the changes in visceral function following administration of insulin are caused by stimulation of the vagus centers through the influence of hypoglycemia. Our experiments provide no additional evidence for or against that view. The occurrence of restlessness and tachycardia in dogs, convulsions in other animals, and of sweating and convulsions in the human indicates that the stimulation is general and is not confined to the vagus or the parasympathetic centers. The increased output of epinephrin which tends to counteract the fall in blood sugar may be taken as a specific indication of stimulation of thoracolumbar sympathetic mechanisms.

In such a situation the effect on the pancreas, which receives both excitatory and inhibitory innervation, could hardly be predicted. It would doubtless represent a summation of antagonistic influences and would probably be readily modified by experimental conditions such as anesthesia and operative trauma. It is not surprising, therefore, that our results in unanesthetized dogs are quite opposite to those previously reported on anesthetized, operated animals. Since they agree with the results obtained in human experiments they probably represent the normal reaction of the pancreatic secretory mechanism to the complex situa-

tion resulting from acute hypoglycemia.

The inhibitory effects of hypoglycemia were manifest in some of our experiments only as a late decrease in volume of pancreatic juice secreted in response to a constant stimulus (secretin). Babkin¹ has suggested that these effects may be due to inability of the pancreas to function normally without an abundant supply of carbohydrate for its metabolic needs. In our experiments the volume, when low, could be promptly restored to normal by slightly increasing the rate of secretin injection. The specific gravity of the juice remained high in these circumstances. Clearly the pancreas was still capable of normal function when adequately stimulated. The decreased response to secretin in these experiments could be attributed to vascular changes or to preponderance of the inhibitory innervation.

Summary and Conclusions. Specific gravity and tryptic activity of dog's pancreatic juice secreted in response to continuous injection of secretin are increased following administration of insulin. The average volume secreted during the first hour after giving insulin is not significantly affected. Inhibitory effects of hypoglycemia described as occurring in anesthetized, operated animals are not readily demonstrated in unanesthetized dogs.

15690

Some Effects of Desoxycorticosterone Acetate on Mice Irradiated with X-rays.*

FRIEDRICH ELLINGER.

From the Laboratory for Experimental Radiation Therapy, Long Island College of Medicine, Brooklyn, N.Y.

Total body irradiation with X-rays produces fatty changes in the livers of irradiated animals.¹ We have recently demonstrated

* Aided by grants from the John and Mary R. Markle Foundation, New York, and the Schering Corporation, Bloomfield, N.J.

¹ Ellinger, F., *Radiology*, 1945, **44**, 241.

² Ellinger, F., *Science*, 1946, **104**, 502.

that the administration of desoxycorticosterone acetate protects the livers of mice irradiated with various lethal doses of X-rays against these fatty liver changes.² It was noted in addition that there was a decrease in the mortality rates produced by the previously used X-ray doses. It is the purpose of this paper to analyze these phenomena

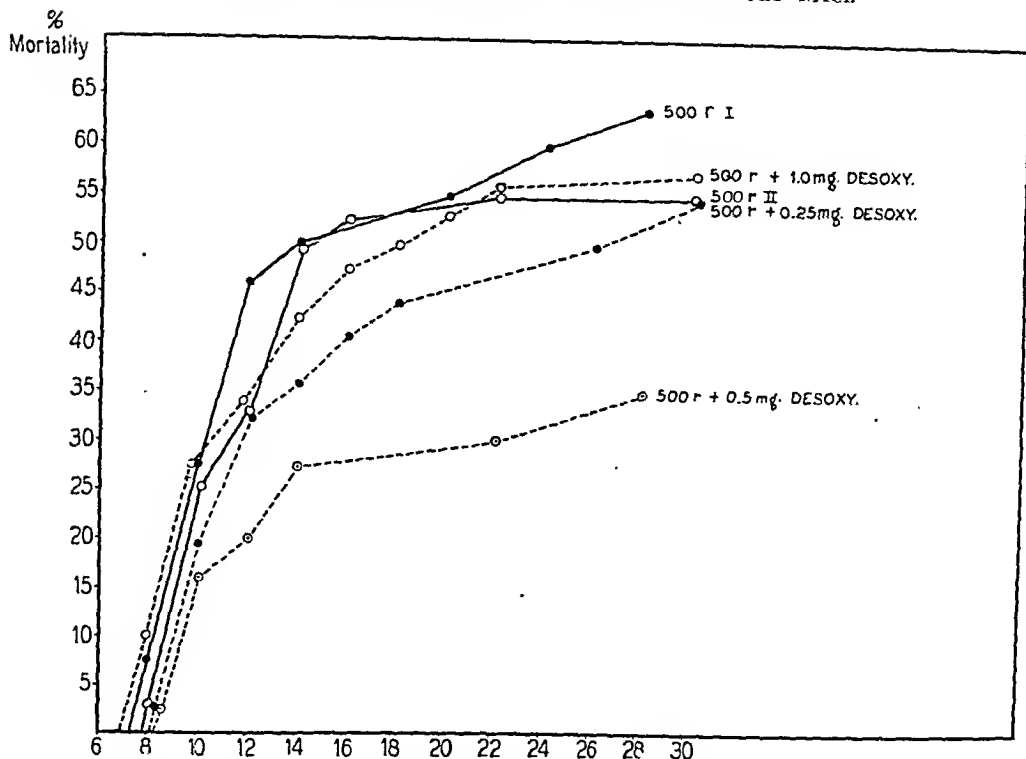


FIG. 1.

Reduction of the mortality rate produced by 500 r/air of X-rays in mice by administration of daily doses of desoxycorticosterone acetate of 0.25, 0.5, and 1.0 mg respectively. Abscissa: days after exposure to X-rays. Ordinate: % mortality.

in more detail.

Methods. A total of 166 white male Swiss mice $22 \text{ g} \pm 15\%$ in weight was used, 62 of these received total body irradiation with X-rays only, while 104 animals received similar irradiation and desoxycorticosterone. All mice received 500 r/air in one exposure (HVL 0.75 mm Cu). This dose represents the LD_{50} . The technic of irradiation was the same as previously described.³

Daily doses of desoxycorticosterone acetate[†] in sesame oil of either 0.25, 0.5 or 1.0 mg were administered intramuscularly 6 times weekly over a period of 10-18 days. The total doses varied between 2.5 and 13.0 mg.

The effect on mortality rate, fat content of livers and radiation changes in other or-

gans was studied at death of the animals. Survivors were observed 28-40 days, in some instances up to 240 days.

The fat content of livers was studied using sections stained with Sudan III, with the following grading:

- 0 No sudanophile fat.
- + Traces of fat, as occasionally seen in non-irradiated livers.
- ++ Increased amount of fat with definite arrangement around central vessel.
- +++ Considerable increase in fat.
- ++++ Fat making up an entire lobulus.

The arithmetical mean of these various grades of sudanophile fat was used for the quantitative evaluation of the histological changes, and called the "fat index."

Results. A graphic presentation of our experiences with various doses of desoxycorticosterone on the lethal effect produced by 500 r/air of X-rays, given as total body irradiation in one exposure, is shown in Fig. 1. It might be noted that desoxycorticosterone

³ Ellinger, F., *Radiology*, 1945, **44**, 125.

[†] We are greatly indebted to Dr. E. Henderson of the Schering Co. for generously supplying us with desoxycorticosterone acetate.

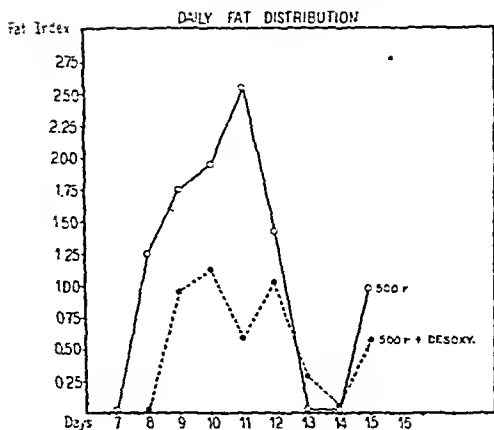


FIG. 2.

Influence of treatment with desoxycorticosterone on the appearance of sudanophile fat in the livers of irradiated mice. Abscissa: Days after exposure to X-rays. Ordinate: Arithmetical mean of the arbitrary grades of sudanophile fat (fat indices) for each day.

decreases the lethal effect of this X-ray dose.

From our previous studies it was inferred that there might be a quantitative relationship between the dose of desoxycorticosterone and the decrease in mortality rate produced

by the X-rays.

Our data on the influence of desoxycorticosterone on the mortality rate have therefore been broken down into 3 groups for a closer analysis of this phenomenon: (a) mice receiving 0.25 mg daily (36 animals), (b) mice receiving 0.5 mg daily (34 animals), (c) mice receiving 1.0 mg daily (34 animals). Furthermore, the total data concerning the mortality rate produced by irradiation of mice with 500 r/air not treated with desoxycorticosterone has been divided into 2 groups: 500 r I and II consisting of 35 and 27 animals respectively, in order to demonstrate the variation in the mortality rate of solely irradiated mice. The graph demonstrates a slight variation in the mortality curves of the 2 groups of mice which received irradiation only. Administration of daily doses of 0.25 mg of desoxycorticosterone decreases the lethal effect of the X-ray dose beyond the variation observed in untreated irradiated mice. The decrease in mortality rate is still more marked in the group of mice which received 0.5 mg of desoxycorticosterone daily. The administration of 1.0 mg, how-

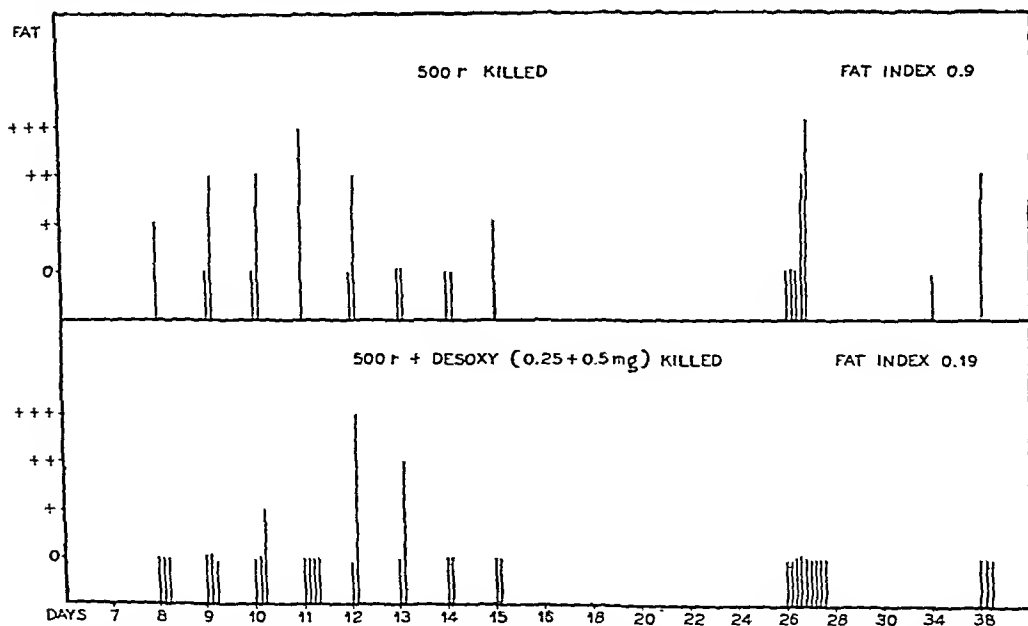


FIG. 3.

A comparison of the fat content of livers in solely irradiated and irradiated and desoxycorticosterone treated animals killed at various days, also indicates reduction in the content of sudanophile fat in the desoxycorticosterone-treated group. Each column represents the findings in one mouse. Abscissa: Days after exposure. Ordinate: Arbitrary grades of sudanophile fat.

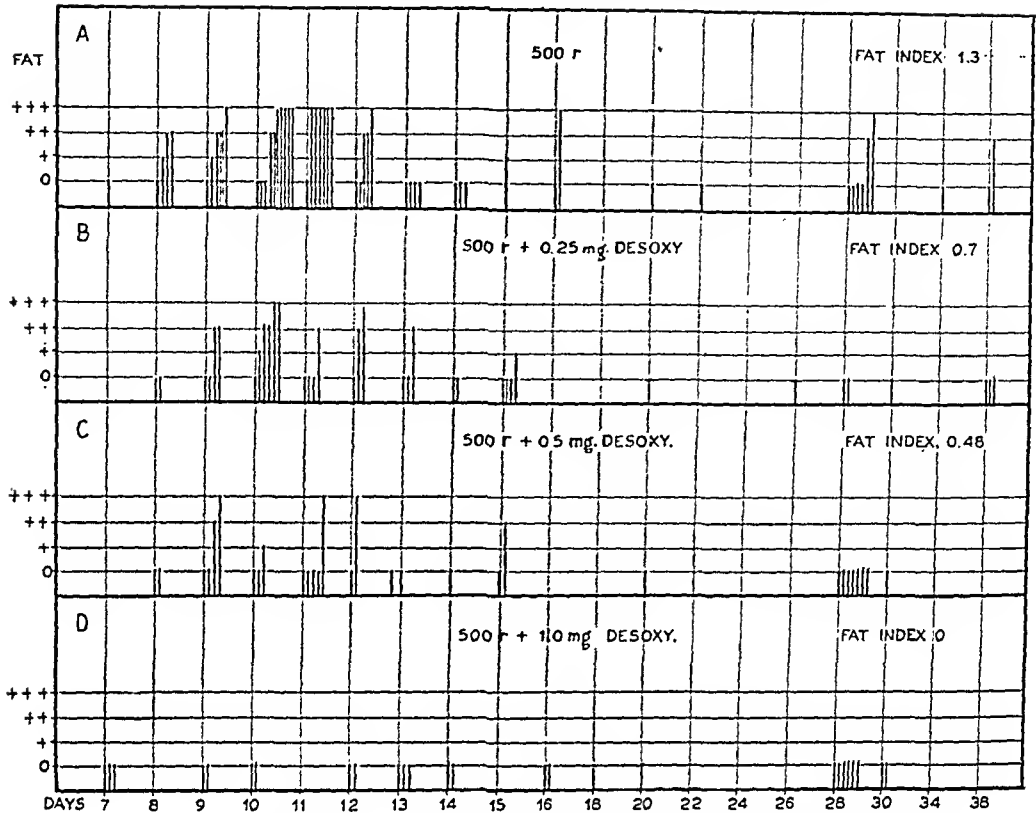


FIG. 4.

Analysis of the influence of the size of the daily dose of desoxycorticosterone acetate on the appearance of sudanophile fat of the livers of irradiated mice, indicates a graded effect of desoxycorticosterone.

ever, does not change the mortality rate. The mice receiving the largest daily doses evidently were suffering from an intoxication produced by these large amounts of desoxycorticosterone, which manifested itself in a ragged fur, sometimes loss of fur and considerable emaciation of the animals.

Our observations concerning the liver changes found in both the untreated irradiated and the irradiated and desoxycorticosterone-treated mice are presented in Fig. 2. This graph shows that the decrease in mortality rate produced by the administration of desoxycorticosterone is accompanied by a marked and statistically valid decrease in the formation of sudanophile fat in the livers of irradiated and desoxycorticosterone-treated animals.

Fig. 2 confirms previous observations¹ that the occurrence of sudanophile fat in irradiated livers proceeds in waves with peaks at

various times after cessation of irradiation.

Recognition of this fact raises the question, whether the observed reduction in the occurrence of sudanophile fat in the irradiated and desoxycorticosterone-treated animals might not be due to the fact that death in these animals occurs at later dates, where the manifestation of sudanophile fat is less pronounced.

To answer this question animals have been killed at various dates after irradiation in both groups. Fig. 3 shows our observations. The data, though not statistically relevant, appear significant, showing a marked reduction in the amount of sudanophile fat in the desoxycorticosterone-treated group, when compared with the finding in the untreated irradiated mice. The fat indices are 0.19 and 0.9 respectively.

In Fig. 4, finally, data on the effect of desoxycorticosterone on the appearance of

sudanophile fat are arranged according to the size of the daily doses of desoxycorticosterone. Section A of Fig. 4 shows the results in the untreated irradiated mice. Section B in those which received 0.25 mg of desoxycorticosterone daily, Section C of those which received 0.5 mg and Section D of those which received 1.0 mg daily. The fat indices are 1.3, 0.7, 0.48 and 0.00 respectively.

The trend of Fig. 4 corresponds to the observations concerning the influence of various daily doses of desoxycorticosterone on the mortality rate. It is very interesting to note that the administration of daily doses of 1.0 mg are able to suppress the appearance of sudanophile fat in the livers completely, although these doses do not reduce the mortality rate.

No significant changes in the effect of the X-ray on spleen and bone marrow was observed with any of the used doses of desoxycorticosterone.

Discussion. The data presented in this paper confirm the previously described protective action of desoxycorticosterone acetate against X-ray-induced liver changes. They establish a parallelism between the reduction in sudanophile liver fat and the decrease in mortality rate in the irradiated and desoxycorticosterone-treated animals.

The analysis of the phenomena presented reveals that both effects within certain limits are dependent on the size of the daily dose of desoxycorticosterone.

The observation of a graded action of desoxycorticosterone with respect to its protective power against X-ray-induced liver damage, seems to indicate a definite antagonistic effect to the processes leading to the appearance of sudanophile fat in the liver.

We have recently adduced the evidence⁴ which makes it highly probable that the occurrence of fatty changes in the livers of irradiated animals is the result of an indirect action of the rays by release of tissue break-down products, histamine-like in character, most probably histamine itself.

Since desoxycorticosterone is known to counteract certain histamine effects⁴ it ap-

pears highly probable that its protection against radiation-induced liver changes is based on this known histamine antagonism.

In favor of this explanation of the effect of desoxycorticosterone is the observation that desoxycorticosterone leaves the radiation effects on spleen and bone marrow unchanged. These effects are considered as predominantly the result of the direct destructive action of the X-ray in these tissues.³

The evidence presented in this paper indicates the possibilities of closer analysis of radiation effects by pharmacological means.

Finally, these data provide a pharmacological basis which justifies the use of desoxycorticosterone in the treatment of radiation sickness, a general intoxication of the irradiated patient, caused by the release of tissue break-down products. While our data indicate in a general way the usefulness of desoxycorticosterone for the treatment of radiation sickness, these observations should not be interpreted as indicating optimal doses for therapeutic purposes.

Summary. 1. A reduction in mortality rate of mice irradiated with X-ray by the use of desoxycorticosterone acetate has been demonstrated. 2. The reduction in mortality rate has been correlated with the protective action of desoxycorticosterone against radiation-induced liver changes. 3. It has been shown that reduction in mortality rate and protection against radiation-induced liver changes are dependent, within limits, on the size of the daily dose of desoxycorticosterone. 4. It has been suggested that the graded effect of desoxycorticosterone is possibly due to an antagonism to the indirect action of the X-rays which consists in the release of histamine-like substances from the irradiated tissues. 5. The data presented are proposed as a pharmacological basis which justifies the clinical use of desoxycorticosterone in the treatment of radiation sickness.

The author wishes to express his gratitude to Dr. A. L. L. Bell, Director of the Department of Radiology, Long Island College of Medicine, for his kind interest in and support of these investigations.

Valuable technical assistance has been rendered by Miss Jean Bernstein.

⁴ Swingle, W. W. and Remington, J. W., *Physiol. Rev.*, 1944, **24**, 89.

Quantitative Measurement of Growth of *Mycobacterium tuberculosis*. Effect of Streptomycin.*†

DOROTHY G. SMITH. (Introduced by Selman A. Waksman.)

From the New Jersey Agricultural Experiment Station, New Brunswick, N.J.

Quantitative estimation of the growth of *Mycobacterium tuberculosis* has been seriously handicapped by lack of simple, accurate, and safe methods of measurement. For this reason, the determination of the normal culture cycle of this organism, the rate of growth, and the *in vitro* effects of growth-promoting and growth-inhibiting substances has been difficult. Visual approximation of growth and weighing of pellicles with all the concomitant danger due to handling of the culture have been the only available, though unsatisfactory, methods. These were dangerous, or they involved technical difficulties and inaccuracies, as pointed out by Mueller.¹

Recently, Youmans² measured the normal culture cycle, growth rate, and generation time of the H37Rv strain of the virulent human type tubercle bacilli by means of micro-Kjeldahl nitrogen determinations. He suggested the use of this procedure in the evaluation of the efficacy of chemotherapeutic agents upon the organism of tuberculosis. Because of the complexity of this test, its use for mass determinations may be questioned.

Dubois³ suggested the possibility of making use of nephelometric measurements in establishing the value of the oil of chaulmoogra and its derivatives on the growth of avian bacilli in Kirchner's medium.⁴ Homogeneous growth in this medium, contrary to the usual result obtained with cultures of

mycobacteria, was reported.

Dubos,⁵ using a modification of the Kirchner formula, developed a medium in which both the pathogenic and the saprophytic varieties of mycobacteria produce a diffuse type of growth. At times, human bacilli form microscopic clumps of loosely packed cells, which can be readily dispersed by moderate shaking to form a uniformly turbid suspension. Such uniformly diffused growth facilitates accurate turbidimetric measurements.

Since none of the existing procedures was satisfactory for safe and accurate determination of growth of mycobacteria, advantage was taken of the uniform turbidity produced in Dubos' medium to develop a suitable method for estimating the growth of these organisms. This method found special application in the study of the effect of streptomycin upon the growth of this organism.

Procedure. Stock cultures of *M. tuberculosis avium* (ATCC No. 7992), an avirulent strain of *M. tuberculosis var. hominis* No. 607, a virulent strain of *M. tuberculosis var. hominis* H37Rv, and a streptomycin-resistant strain of *M. tuberculosis var. hominis* H37Rv obtained from Dr. G. P. Youmans were maintained on glycerol-nutrient agar slants and in Dubos' medium.⁵ For the growth of virulent human varieties, 0.2% bovine serum albumin (fraction V) was added to the medium. The ages of the cultures varied depending on their rate of growth, namely, 7 days for *M. avium* and *M. tuberculosis* No. 607 and 3 to 5 weeks for *M. tuberculosis* H37Rv and H37Rv streptomycin-resistant.

For purposes of inoculation, a homogeneous suspension of cells was made in a simplified modification of Dubos' medium, which was incapable of supporting significant growth and consisted only of the salt mixture plus

* Journal Series Paper, New Jersey Agricultural Experiment Station, Rutgers University, Department of Microbiology.

† Partly supported by a grant made by the Albert and Mary Lasker Foundation of New York.

¹ Mueller, J. H., *J. Bact.*, 1935, **29**, 383.

² Youmans, G. P., *J. Bact.*, 1946, **51**, 703.

³ Dubois, A., *Ann. Soc. belge de Med. Trop.*, 1944, **24**, 1.

⁴ Kirchner, O., *Zentral. f. Bakteriologie*, 1932, I Orig., **124**, 403.

⁵ Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

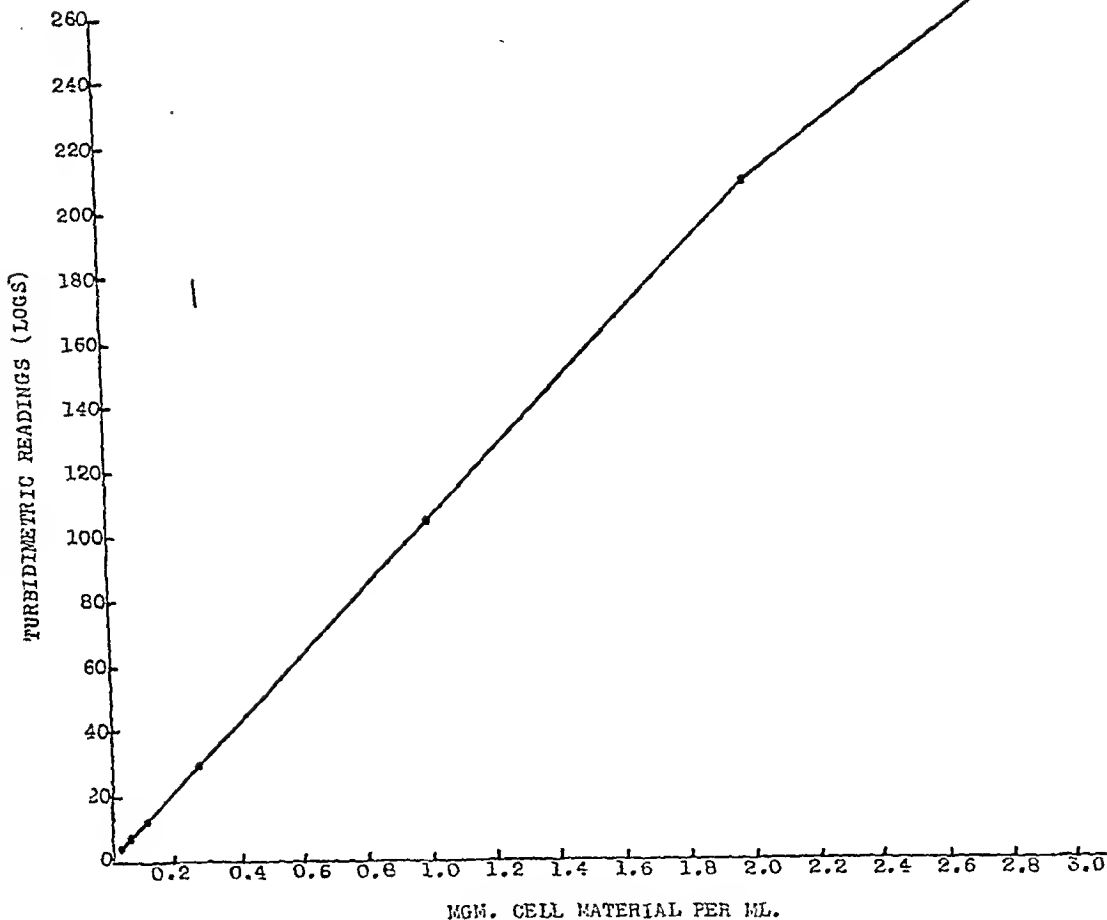


FIG. 1.

Turbidimetric Measurement of Suspensions of *Mycobacterium tuberculosis* No. 607.

Tween 80.† The purpose of this Tween-salt solution was to avoid, as much as possible, the introduction of nutrients when inoculating nutritionally-deficient media.

The suspension was prepared as follows: Growth from an agar slant was added to a small amount of the Tween-salt mixture and shaken with sterile glass beads. This resulted in a homogeneous suspension which could be diluted to the desired density. If cultures grown in Dubos' medium were used, they were diluted or were used undiluted, depending upon their density. Turbidimetric

readings of suspensions containing weighed amounts of cells have shown that 1 mg of cells (wet weight) per milliliter of fluid formed a suspension which gave turbidimetric readings of 100-105 on a Klett-Summerson colorimeter. A certain amount of growth of the organism was suspended in the medium and adjusted to give a reading of 100. Further dilution was made with the Tween-salt solution until the required concentration of cells was obtained.

The experiment was now set up by seeding the media with 4.5 ml portions of a suspension of bacterial cells in test tubes. For controls, 5-ml amounts were used; for the study of the effect of streptomycin, 0.5-ml portions of the various concentrations of drug

† Tween 80 is a polyethylene derivative of sorbitan monooleate and acts as a detergent in this medium.

TABLE I.
Influence of Inoculum on Rate of Growth of *Mycobacterium tuberculosis* No. 607.*

Time of incubation, hr	Concentration of cells, mg/ml				
	10 ⁻³	10 ⁻⁵	10 ⁻⁷	10 ⁻⁹	10 ⁻¹⁰
	Turbidimetric readings (in logs)				
24	9	7	0	0	0
48	26	7	0	0	0
72	44	34	8	0	0
96	60	54	31	8	0
184	106	100	83	72	0
330	150	159	138	153	0

* Dubos' medium without enrichment with dextrose, vegex, and albumin.

were added to 4.5 ml of the seeded medium. The cultures were incubated at 37°C. and turbidimetric readings were made at frequent intervals. In media with extremely low nutritive value, some firm clumps of cells were formed after 10 to 21 days of incubation, depending on the rate of growth of the culture. No clumping was observed in Dubos' medium even after 6 weeks' incubation.

The tubercle bacilli can be grown on fairly simple media. Dubos' medium, consisting of KH_2PO_4 , $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, sodium citrate, Tween 80, an enzymatic digest of casein, dextrose, vegex (a vegetable extract), and bovine serum-albumin, is rather complex and supports abundant growth of the human pathogenic strains of mycobacteria as well as of the saprophytic forms. However, considerable growth can still be obtained if all complex organic materials are omitted and ammonium salt is used as a source of nitrogen with the citrate as the sole source of carbon. Thus the above medium can be simplified so as to be made synthetically, and still remain adequate for growth of the organism. By the use of this

or similar modifications, it was possible to study the physiology of the tubercle bacilli and to investigate the effect of streptomycin on the metabolism of the organism.

In preliminary experiments, bacterial suspensions were prepared for purposes of inoculation using weighed amounts of cells. The uniform turbidity of these suspensions made possible the construction of a standard curve for the determination of the weight of the cell material in terms of turbidity readings. This is shown in Fig. 1, where the weight of the cells ranging from 0.01 mg/ml to 2.0 mg/ml was plotted against turbidimetric readings as given on the scale of the Klett-Summerson colorimeter. Readings above 2.0 mg/ml diverged from a straight line, whereas those below 0.01 mg/ml could not be accurately measured. Two advantages of such a procedure are obvious, namely, the ease of correlating turbidity with the weight of cell material, and the elimination of undue handling of the culture.

If a standard curve is made based on the plate counts of viable bacteria in a suspension, the value of the turbidimetric readings

TABLE II.
Influence of Nitrogenous Materials on the Rate of Growth of *Mycobacterium tuberculosis* var. *hominis* H37Rv.

Time of incubation, hr	Concentration of cells in mg/ml, 5×10^{-3}			
	Constituent of medium			
	NH_4 citrate, 0.2%	Asparagine, 0.2%	Tryptophane, 0.1%	Dubos' medium (albumin)
Turbidimetric readings (in logs)				
24	0	0	2	0
72	0	2	8	3
120	0	4	13	16
244	9	16	20	56
334	11	19	26	85
507	11	13	34	244

TABLE III.

Influence of Nitrogenous Materials on the Bacteriostatic Action of Streptomycin. *Mycobacterium tuberculosis* No. 607*.

Source of nitrogen	Streptomycin, μg/ml	Incubation in hr					Final pH
		24	48	72	187	312	
		Turbidimetric readings (in logs)					
NH ₄ citrate, 0.2%	0.4	0	17	27	27	67	6.20
	0.2	0	11	27	25	67	6.20
	0.1	1	16	27	30	72	6.20
	0	1	14	29	30	60	6.20
Asparagine, 0.2%	0.4	0	0	0	0	5	7.88
	0.2	0	0	0	30	45	7.92
	0.1	0	16	37	35	31	8.05
	0	2	25	38	37	85	8.06
Casein digest, 0.2%	0.4	0	0	0	0	0	8.20
	0.2	2	2	3	6	6	8.18
	0.1	2	16	34	83	108	8.30
	0	3	13	43	113	154	8.40
Beef extract, 0.3%	0.4	0	0	0	52	155	7.88
	0.2	1	10	25	96	157	8.38
	0.1	4	27	48	150	165	8.48
	0	5	31	55	165	150	8.45

* The media were seeded with 10-5 mg cells/ml.

may be extended to correlate turbidity measurements with the number of viable cells. A series of 10 determinations were made with 7-day-old cultures of *M. tuberculosis* No. 607 and of *M. avium* on glycerol-nutrient agar. Consistently reproducible counts of viable cells were obtained, the numbers of viable organisms being 50.6 million cells for *M. tuberculosis* and 59.5 millions for *M. avium* per milligram, with a turbidity reading of 101, with 1 mg of cell material per milliliter. When determining numbers of viable organisms, certain limitations must be kept in mind. First, at least on solid medium, it is often difficult to initiate growth with small numbers of tubercle bacilli; wide variation in results may, therefore, occur. Secondly, optical densities are proportional to the numbers of bacteria only where organisms of the same size or size distributions occur.⁶ When grown in the presence of antibiotic agents, the organisms frequently assume elongated shapes, increasing in volume rather than dividing into separate components. In comparing cultures grown in the presence and in the absence of antibiotic agents, the

optical density is a measure of the relative volumes or masses rather than of the numbers of bacteria. Even with these limitations, turbidimetric readings were found to permit satisfactory interpretation of results once the relationship between turbidity, weight of cells, and numbers of viable organisms had been established.

Experimental Results. The application of the turbidimetric method for the quantitative estimation of the growth of tubercle bacilli is demonstrated in several experiments. Table I shows the influence of inoculum on the rate of growth of *M. tuberculosis* No. 607 in a modification of Dubos' medium. During the first 96 to 184 hours, the rate of growth was dependent upon the size of the inoculum; then growth appeared to proceed at a more or less uniform rate regardless of the initial concentration of the cells, as indicated by the readings at 330 hours. Similar results were obtained with *M. avium* and with the streptomycin-resistant and -sensitive strains of *M. tuberculosis* H37Rv. In this case, as in all other media used for the growth of tubercle bacilli, the strains pathogenic to man grew at a much slower rate than the saprophytes. The data also agree with the plate counts of viable bacteria, growth taking place

⁶ Treffers, H. P., *Yale J. of Biol. and Med.*, 1946, 18, 609.

with 10^{-9} mg of cell material per milliliter but not with 10^{-10} . According to the actual count, 10^{-7} mg of cell material should contain 6 viable cells; it is, of course, possible that one or more cells would be found even in the higher dilutions which would account for the growth recorded for the 10^{-9} mg/ml suspension.

When a medium is inoculated with increasing concentrations of cells, there is a corresponding decrease in the lag period of growth, but all of the resulting cultures finally reach the same degree of density. On the other hand, if the inoculum is held constant and the nutrient value of the medium is varied, quite different results are obtained. An increase in the amount of growth is found to parallel enrichment of the medium. This is accompanied by a shortening of the lag period.

The effect of nitrogenous materials on the rate of growth of *M. tuberculosis var. hominis* H37Rv is presented in Table II. The incubation time required for the initiation of growth in a medium containing ammonium citrate was 10 times that required in one containing casein, a vegetable extract and serum-albumin. The difference in the amounts of growth was even more striking, the more complex medium giving 20 times more abundant growth. The rate and amount of growth are dependent not only on the num-

ber and concentration of nutrients but on the nature of the nutritive material. Tryptophane in a concentration of 0.1% is a better nutrient than 0.2% asparagine for all of the strains of mycobacteria.

Having established the normal rate of growth of mycobacteria, it was now possible to evaluate the effect of streptomycin upon the growth of mycobacteria in the presence of different sources of energy. Regardless of the source of nitrogen or of the amount of growth supported by the medium, little difference was found in the amount of streptomycin required for bacteriostasis, as shown in Table III. The only exception is found in the ammonium citrate medium; limited effectiveness of streptomycin is possibly due to the high acidity of this medium resulting from the growth of the organism.⁷ Similar results were obtained with *M. avium* and the human pathogenic varieties.

Summary. A simple, safe, and accurate method is described for the quantitative estimation of the growth of *Mycobacterium tuberculosis* based upon turbidimetric measurements in Dubos' medium.

This procedure can be applied to measuring the rate and amount of growth of *M. tuberculosis* in various media, as affected by the presence of streptomycin.

⁷ Waksman, S. A., Bugie, E., and Schatz, A., *Proc. Staff Meetings of Mayo Clinic*, 1944, 19, 537.

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Preparation of an Anti-Ulcer Factor from Human Urine.*

ARNE N. WICK, ALICE JEAN IRISH, FRANCES PAULS, AND EATON M. MACKAY.

From the Scripps Metabolic Clinic, La Jolla, California.

The preparation of crude extracts from urine (Urogastrone) and hog intestinal mucosa (Enterogastrone) which possess anti-gastric secretory and antiulcer activity in dogs, such as the Mann-Williamson and the Pavlov preparation has been reported in the

literature. Two methods have been applied to urine for the preparation of Urogastrone or Urogastrone-like substances. Necheles¹ has prepared an inhibitor of gastric secretion and motility by treating normal human urine with ammonium sulphate and fractionating the resulting precipitate between vary-

* The authors are indebted to Mr. and Mrs. Francis E. Fowler and members of their family whose generosity has made these studies possible.

¹ Necheles, H., Hanke, M. E., and Fantl, E., *Proc. Soc. Exp. Biol. and Med.*, 1939, 42, 618.

ing concentrations of alcohol. Gray, Wieczorowski and Ivy² used a benzoic acid adsorption method for the preparation of an anti-secretory substance from normal male urine, and Friedman *et al.*³ have found an inhibitory substance in normal female urine by using the same method of adsorption. An examination of the methods elicits little information regarding the chemical properties of the extracts except that they are water soluble and insoluble in organic solvents such as ethyl ether, benzene, and petroleum ether.⁴ The further characterization of these extracts has been greatly retarded due to the lack of a convenient assay method.

The Mann-Williamson dog which has been generally used in testing for antiulcer properties of various preparations, entails considerable time, expense, and experience which greatly limits its usefulness. For an excellent discussion regarding the present status of these antiulcer "hormones" see the reviews of Ivy⁵ and Sandweiss.⁶

With the recent availability of the rat assay method^{7,8} in which extensive gastric ulceration can be produced in the rat in the short period of 7-9 hours following ligation of the pylorus, the investigation of antiulcer factors has been continued. A fraction has been prepared from human urine which when given to rats in sufficient quantity at the time of pyloric ligation prevents the formation of the gastric ulcers. The method consists essentially of adsorbing the activity on carbon from clarified urine at neutral pH, eluting the activity from the carbon with acidified aqueous-acetone at pH 2, and precipitating the activity from the eluate by in-

creasing the acetone concentration. This method has been used continuously in this laboratory over a period of 6 months without obtaining an inactive preparation.

Experimental. The urine used in these studies was obtained over 24-hour periods from male and female patients who were in the clinic mainly for diagnostic purposes. Most of this urine was collected in the early morning hours and processed within 8 hours. The urine, which was collected in 2.5 liter bottles and preserved with chloroform, was decanted from the chloroform before carrying out the initial clarification with Celite-545.

The Assay procedure used in these experiments has been mentioned elsewhere.⁸ The method consists of using male rats 8 weeks of age or female rats 10 weeks of age weighing 120 to 150 g. The animals are fasted for 48 hours before operation in individual cages with wide wire mesh bottoms, supported well above the dropping paper. Water is allowed *ad libitum*. For the operative procedure the rats are anesthetized with ether. Through a short midline incision extending downward from the xiphoid process, the duodenum is grasped lightly with ring forceps and a ligature of linen thread is placed exactly at the pyloric sphincter. The incision is closed with Michele wound clips and the wound is covered with a thin coating of flexible collodion. At the time of the operation the material to be tested for antiulcer activity is administered intravenously to the test group and physiological saline is given to the control group. A minimum of 6 to 10 rats should constitute a test group and an equal number is used for the control group. Eight or 9 hours after tying off the pylorus the animals are sacrificed. The abdominal cavity is opened, when the animal is under ether anesthesia, and the esophagus is grasped with a hemostat. The stomach is then freed of mesentery and removed. A small slit is made on the greater curvature of the distended stomach for collection of the gastric juice. The stomach is finally cut along the whole length of the greater curvature and after a brief rinsing in physiological saline, is stretched out with the aid of pins on beeswax for examination

² Gray, J. S., Wieczorowski, E., and Ivy, A. C., *Science*, 1939, **89**, 489.

³ Friedman, M. H. F., Recknagel, R. O., Sandweiss, D. J., and Patterson, T. L., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 509.

⁴ Gray, J. S., Wieczorowski, E., Wells, J. A., and Harris, S. C., *Endocrinology*, 1942, **30**, 129.

⁵ Ivy, A. C., *Fed. Proc.*, 1945, **4**, 222.

⁶ Sandweiss, D. J., *Gastroenterology*, 1945, **5**, 404.

⁷ Shay, H., Komarov, S. A., Fels, S. S., Meranze, D., Gruenstein, M., and Siplet, H., *Gastroenterology*, 1945, **5**, 43.

⁸ Pauls, F., Wick, A. N., and MacKay, E. M., *Science*, 1946, **103**, 673.

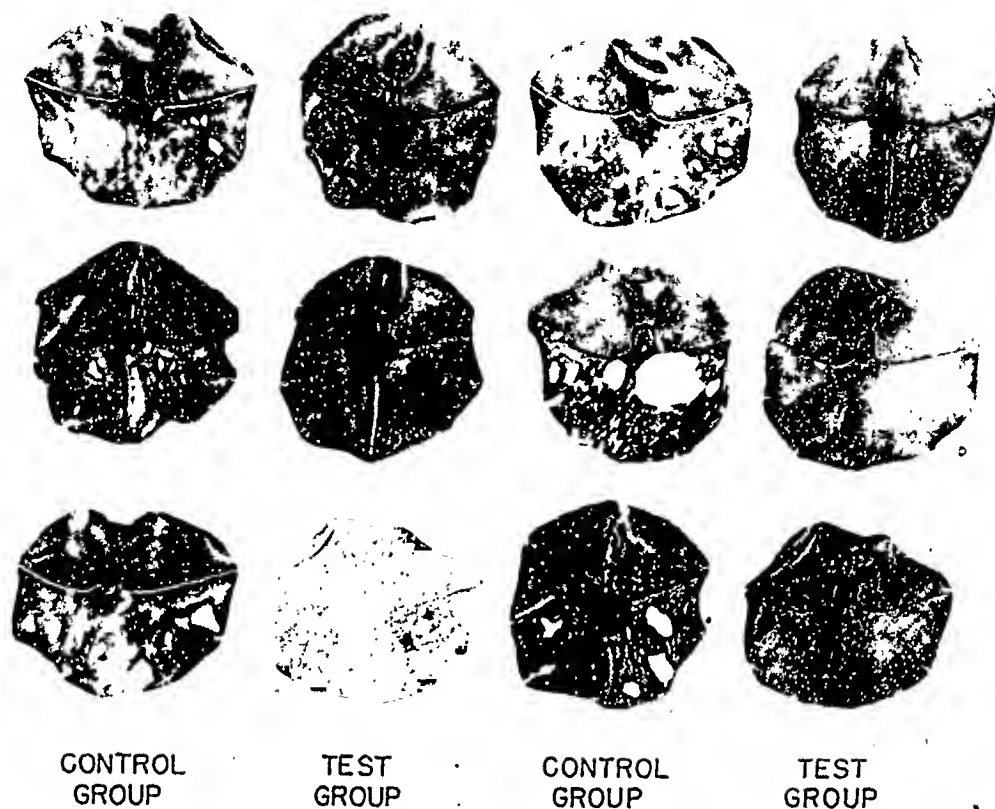


Fig. 1.

of the ulceration.

The steps for the preparation of the anti-ulcer substance are described for 100 liters of urine. Together with the distribution of solids they are outlined in detail in the accompanying flow sheet. The routine used in this laboratory is to pool all of the urine each morning and to clarify it by filtration with the aid of 1% Celite. The pH of the clarified urine has ranged from 6.0 to 6.5 and no further adjustment is made before the addition of the carbon. The percent Norite S.G. has been found to give satisfactory results for the adsorption of the activity. After the suspension is stirred for 20 minutes, the carbon is collected in large Buchner funnels. Celite is added to the mixture before filtering in order to facilitate the filtration procedure. The charcoal cakes thus obtained each day are stored in 4°C

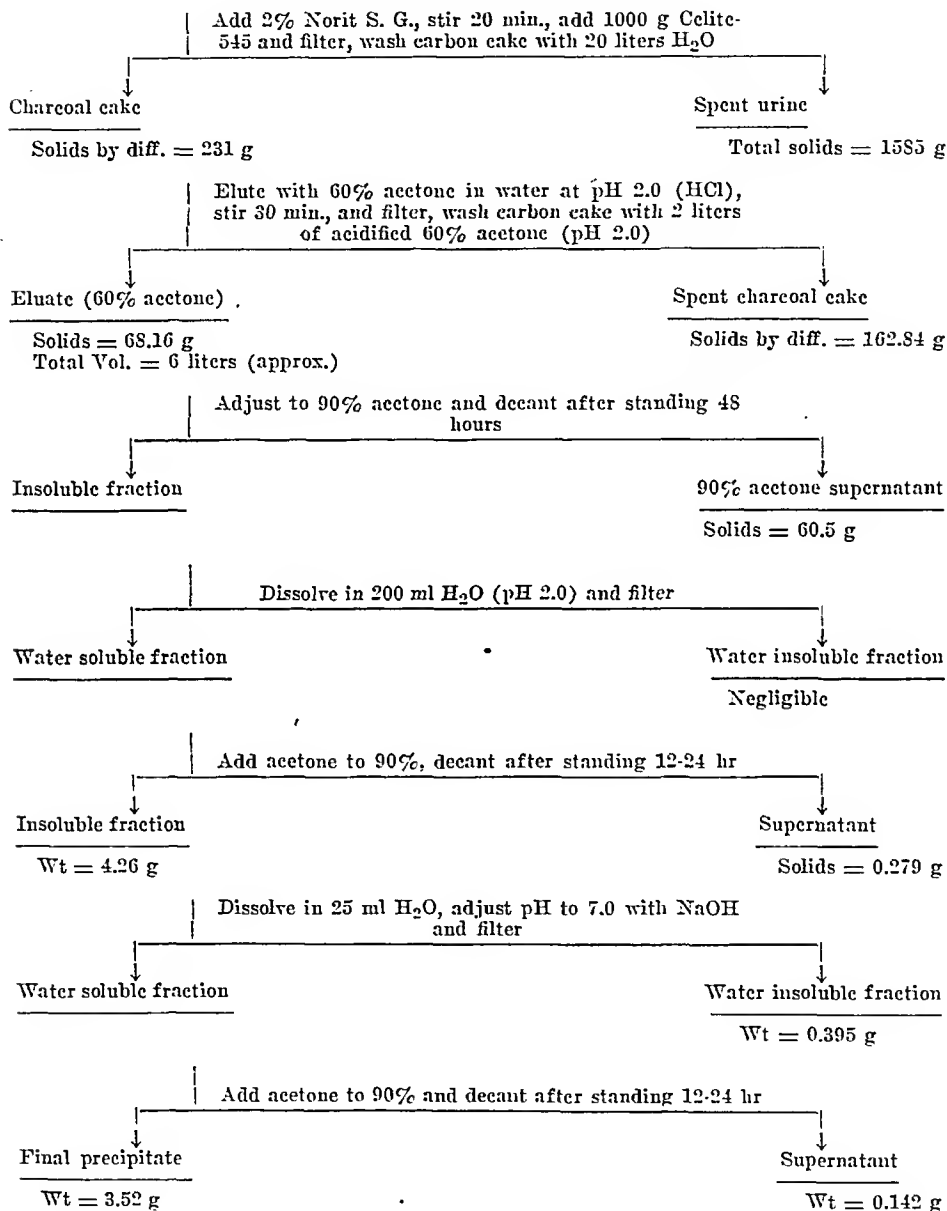
cold room until the end of the week at which time they are combined for the elution of the active substance. The elution is carried out in 60% acetone by volume in water at pH 2.0. The water content of the carbon cake, which is readily determined by weighing, is taken into account when adjusting to the 60% acetone concentration. Additional amounts of 60% acetone in water are added if necessary to prepare a thin slurry for efficient stirring. Although one elution is carried out in these experiments, a second and third elution can be used if so desired. The final product, after the neutralization and the subsequent filtration, can be obtained by the lyophile process or by increasing the acetone concentration as shown in the flow sheet.

Results. The preparation obtained in this carbon-acetone method will prevent complete-

FLOW SHEET FOR THE ISOLATION OF AN ANTI-ULCER EXTRACT FROM URINE.

Vol. of clarified starting urine = 100 liters.

Total solids = 1816 g.



ly the formation of gastric ulcers in our rat-assay method. The quantity of material required to inhibit the ulceration has varied in different preparations from 50 to 100 mg/rat. Fig. 1 shows the stomachs removed from 6 control rats which received injections of physiological saline, and 6 test animals which received 50 mg of the urinary product intravenously at the time of pyloric ligation. The control group all show marked ulceration, while those in the test group receiving the antiulcer substance show a marked diminution or a complete absence of ulceration. Such clear cut results are not always obtained, especially when the minimal active dose of antiulcer material is being investigated. For such cases we have been recording the gastric ulceration by an arbitrary scale which ranges from "clear" or "zero" to "4 plus" ulceration.

Discussion. The chemical nature of the urinary antiulcer factor described here has not been determined, although the carbon-acetone method, which we have used for its preparation, is similar to that used for the isolation of the basic substance streptomycin.⁹ A discussion of the chemical relationship between Urogastrone, Enterogastrone, and the urinary "carbon-acetone" material and proof that the antiulcer activity of the "carbon-acetone" produced extract is due to a single chemical entity must wait until further purification can be carried out.

Our urinary line product is approximately 5% ash and contains 5-6% nitrogen. The neutralized material is insoluble in organic solvents which are immiscible with water. The product obtained by acidifying a solution of the antiulcer factor to pH 2.0 and drying by the lyophile process is soluble to the extent of approximately 1 g per 100 ml methyl alcohol at room temperature. This fraction has greater activity than the starting material or the methyl alcohol insoluble fraction. The antiulcer activity of the crude

line product can be more readily increased by fractionating with aqueous-acetone. In a 5% aqueous solution of starting material, the fraction precipitated by increasing the acetone concentration from 30 to 60% contains 30% of the solids and approximately 60% of the starting activity.

The antiulcer factor is fairly stable to alkaline hydrolysis. When 800 mg is dissolved in 20 ml of 0.1 N NaOH and refluxed for 24 hours, approximately 50% of the activity is retained. In contrast, when an equal quantity of the material is dissolved in 20 ml of 0.1 N HCl and refluxed for one hour, most if not all of the activity is destroyed. Shorter periods of acid hydrolysis have not been studied although the acid solution darkens rapidly after the start of refluxing.

We are unable at the present time to estimate the concentration of the antiulcer factor normally occurring in the urine. Assays carried out on the clarified and spent urine at maximum tolerable levels (200 mg/rat) have shown no reduction in gastric ulceration. The "90% acetone supernatant" which constitutes 85 to 95% of the solids in the "Eluate" has slight activity at 100 mg/rat. This fraction has an ash content of 28% and since we have observed that it is quite toxic at therapeutic doses, we are hesitant in attributing much importance to this fraction.

No attempt can be made at this time to speculate on the mechanism of the antiulcer effect exerted by the material prepared from urine. Ulceration may be reduced in extent or entirely prevented by a reduction in the secretion of gastric juice, impaired peptic activity of this juice or an enhancement of the resistance to ulceration which is inherent in the gastric mucosa.

Summary. A neutral extract has been prepared from normal human urine by adsorption on charcoal and elution thereof with 60% acetone in water at pH 2. This material, when given to rats at the time of pyloric ligation, prevents the development of the gastric ulceration which normally occurs with this procedure.

⁹ Vander Brook, M. I., Wick, A. N., De Vries, W. H., Harris, R., and Cartland, G. F., *J. Biol. Chem.*, 1946, **165**, 463.

Antagonism of the Hemorrhagic Syndrome Induced by Derivatives of 3-Hydroxy-1,4-Naphthoquinone.*

CARL C. SMITH. (Introduced by L. H. Schmidt.)

From the Institute of Medical Research, The Christ Hospital, Cincinnati, Ohio.

It has been reported recently¹ that certain 2-substituted-3-hydroxy-1,4-naphthoquinones, when administered to the white rat, induce a hemorrhagic syndrome which is associated with a hypoprothrombinemia. Preliminary studies showed that the hemorrhagic activities of at least one of these naphthoquinones, 2-(3-cyclohexylpropyl)-3-hydroxy-1,4-naphthoquinone (SN 5090[†]), could be antagonized completely by simultaneous administration of vitamin K₁. Similar administration of 2-methyl-1,4-naphthoquinone had less marked effect. Subsequent to this work experiments were carried out on 2 other 2-substituted-3-hydroxy derivatives. When it was found that these compounds, like the naphthoquinones described previously,¹ also induced a hemorrhagic syndrome and hypoprothrombinemia it was decided to expand the previous observations on the antagonistic action of vitamin K₁ and 2-methyl-1,4-naphthoquinone. The present report deals primarily with this phase of the study.

Experimental. The compounds studied included 2-cyclohexyl-3-hydroxy-1,4-naphthoquinone (SN 5094), 2-(2-methyloctyl)-3-hydroxy-1,4-naphthoquinone (SN 5949), and 2-[3-(*p*-phenoxyphenyl)propyl]-3-hydroxy-1,4-naphthoquinone (SN 13,936[‡]). All 3 of these naphthoquinones induced a hemorrhagic syndrome characterized by gross ex-

travasations of blood into the subcutaneous tissues of the limbs, head, neck and scrotum, and diffuse bleeding of the gastrointestinal mucosa; associated with this syndrome were greatly prolonged clotting and prothrombin times. SN 5094 was the least toxic of the compounds; daily doses of 400 mg per kg uniformly produced fatal lesions but doses of 200 mg had little effect. SN 5949 was somewhat more toxic; it usually produced fatal lesions at 200 mg per kg but had little effect at lower doses. SN 13,936 was the most toxic of the compounds; it uniformly was fatal at 200 mg per kg doses and produced severe hemorrhages at doses of 100 mg.

The technics employed in studying the antagonistic effects of vitamin K₁ and 2-methyl-1,4-naphthoquinone were similar to those used in the previous study.¹ Male rats, 4 to 5 weeks old, Sprague-Dawley strain, were used throughout and were fed a diet of Purina Dog Chow checkers *ad libitum*.

The drugs and vitamin adjuncts were suspended or dissolved in olive oil and were administered once daily via stomach tube. Treatments were continued for 8 to 12 days where the rats survived that long. Whenever it was possible to obtain blood samples, prothrombin times were measured. Determinations were carried out on whole blood using the method of Kato² with Russell viper venom as the source of thromboplastin. The results of the pertinent experiments have been summarized in Table I.

In the experiments with SN 5094, daily doses of vitamin K₁ of 10 mg per kg completely prevented the development of the hemorrhagic syndrome. Similar doses of 2-methyl-1,4-naphthoquinone were somewhat less effective. Hemorrhagic lesions appeared on the 4th day of the experiment but appeared to regress with continued treatment. At the end of the experiment normal pro-

* The work described in this paper was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Institute of Medical Research, The Christ Hospital, Cincinnati, Ohio.

¹ Smith, Carl C., Fradkin, R., and Lackey, M. D., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61**, 398.

[†] The numbers cited here are those assigned to the compounds by the Survey of Antimalarial Drugs, Baltimore, Md.

[‡] The drugs were prepared by Dr. Louis F. Fieser, Harvard University, and the Abbott Laboratories, to whom we are greatly indebted.

² Kato, K., *Am. J. Clin. Path.*, 1940, **10**, 147.

TABLE I.
The Effectiveness of 2-Methyl-1,4-Naphthoquinone and Vitamin K₁ on the Hemorrhagic Activities of SN 5094, SN 5949, and SN 13,936.

Rat No.	Daily treatment, mg/kg body wt	Days treatment	Effects of treatment	
			Prothrombin time, sec	
1	400 mg SN 5094	4	—	Died 4th day; typical hemorrhagic lesions.
2		4	—	" " " " " "
3		5	—	" 5th " " " "
4		6	—	" 6th " " " "
5		8	—	" 8th " " " "
6	400 mg SN 5094 and 10 mg 2-methyl-1,4- naphthoquinone	6	—	" 6th " " " "
7		11	30	Hemorrhagic lesions on limbs.
8		11	23	" " " " " "
9		11	17	Regressing hemorrhagic lesion in serotum.
10	400 mg SN 5094 + 10 mg vit. K ₁	11	20	No hemorrhagic lesions.
11		11	18	" " " " " "
12		11	17	" " " " " "
13		11	14	" " " " " "
14	200 mg SN 5949	3	—	Died 3d day; no hemorrhagic lesions.
15		5	25*	Hemorrhagic lesions on limbs and head.
16		8	190	" " " " " " serotum.
17		8	50	No hemorrhagic lesions.
18	200 mg SN 5949 and 10 mg 2-methyl-1,4- naphthoquinone	4	—	Died 4th day; no hemorrhagic lesions.
19		8	35	Regressing hemorrhagic lesions on limbs.
20		8	30	No hemorrhagic lesions.
21		8	25	" " " " " "
22	200 mg SN 5949 and 10 mg vit. K ₁	8	15	" " " " " "
23		8	12	" " " " " "
24		8	11	" " " " " "
25		8	10	" " " " " "
26	200 mg SN 13,936	3	—	Died 3rd day; gastrointestinal hemorrhages.
27		4	—	" 4th " " typical hemorrhagic lesions.
28		5	>600	" 5th " " " " "
29		6	—	" 6th " " " " "
30		8	>600	" 8th " " " " "
31	200 mg SN 13,936 and 10 mg 2-methyl-1,4- naphthoquinone	3	—	" 3rd " " gastrointestinal hemorrhages.
32		6	—	" 6th " " typical hemorrhagic lesions.
33		9	—	" 9th " " " " "
34		10	—	" 10th " " " " "
35	200 mg SN 13,936 and 10 mg vit. K ₁	11	30	No hemorrhagic lesions.
36		11	40	" " " " " "
37		11	60	" " " " " "
38		11	140	" " " " " "
39	200 mg SN 13,936 and 100 mg vit. K ₁	12	12	" " " " " "
40		12	14	" " " " " "
41		12	15	" " " " " "
42		12	18	" " " " " "

* Prothrombin time obtained on 7th day. Animal too ill for treatment after 5th day.

thrombin times were obtained.

The findings with SN 5949 were similar to those just described. Vitamin K₁ in 10 mg per kg doses prevented the development of hemorrhagic lesions and hypoprothrombinemia

whereas only a partial effect was obtained with this dosage of 2-methyl-1,4-naphthoquinone.

In the experiments with SN 13,936, 10 mg doses of 2-methyl-1,4-naphthoquinone were

without apparent effect on the syndrome. Even vitamin K₁ at 10 mg per kg doses did not completely block the action of SN 13,936 for, although hemorrhagic lesions were absent, a significant hypoprothrombinemia was present. At 100 mg per kg doses, however, vitamin K₁ blocked development of both the hemorrhagic lesions and the hypoprothrombinemia.

Discussion. The data presented above demonstrate that, as in the case of SN 5090,[†] vitamin K₁ and to a lesser extent 2-methyl-1,4-naphthoquinone antagonize the hemorrhagic syndrome and hypoprothrombinemia induced by SN 5094, SN 5949 and SN 13,936. This finding would seem to strengthen the suggestion that the 2-substituted-3-hydroxy-naphthoquinones exert their hemorrhagic activity by competing with the K vitamins in whatever processes these substances serve in prothrombin formation.

This suggestion appears to offer the only tenable explanation for the hemorrhagic activity of the above naphthoquinones. The compounds do not produce severe liver injury, nor are they especially active antibacterial agents which might interfere with the synthesis of K vitamins by intestinal bacteria. Furthermore, extensive *in vitro* experiments indicate that they do not inactivate

prothrombin as do the indandiones.³ It appears, therefore, as if the relationship of the 2-substituted-3-hydroxy-1,4-naphthoquinones to vitamin K affords another example of antagonism of metabolites by structurally related compounds.

Summary. The development of the hypoprothrombinemia and hemorrhagic lesions, which occurred in the albino rat as the result of ingestion of various 2-substituted-3-hydroxy-1,4-naphthoquinones, could be prevented either partially or completely by simultaneous administration of 2-methyl-1,4-naphthoquinone or vitamin K₁. The amounts of these K vitamins required to block development of the above syndrome differed with the various hydroxy-naphthoquinone derivatives. In each instance, however, vitamin K₁ proved more effective than 2-methyl-1,4-naphthoquinone.

Observations made in this report confirm our earlier suggestion that the hemorrhagic syndrome, which results from administration of 2-substituted-3-hydroxy-1,4-naphthoquinones, is due to competition between these substances and the K vitamins in processes involved in prothrombin formation.

³ Kabat, H., Stohliman, E. F., and Smith, M. I., *J. Pharm. and Exp. Therap.*, 1944, **80**, 160.

15694

Action of 3,3' Methylenebis (4-Hydroxycoumarin) (Dicumarol) on Thromboplastic Activity of Rabbit Brain.*

M. STEFANINI[†] AND M. C. BLANCHER.[‡] (Introduced by Armand J. Quick.)

From the Department of Biochemistry, School of Medicine, Marquette University, Milwaukee, Wisconsin.

Several observations suggest that other factors besides the interference with the formation of prothrombin¹ must be consid-

ered in the pathogenesis of the bleeding tendency produced by dicumarol. Widespread dilatation of capillaries, arterioles and venules has been found, indicating that the hemorrhagic condition is due, at least in part,

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[†] Assistant, Medical Clinic of the University of Rome; at present, Research Fellow, Institute of International Education.

[‡] Research Fellow, Department of Biochemistry,

School of Medicine, Marquette University, Milwaukee, Wisc.

¹ Quick, A. J., *The Hemorrhagic Diseases*, pp. 284-285, C. C. Thomas, Springfield, Ill., 1942.

TABLE I.
Influence of Dicumarol Poisoning on Thromboplastic Activity of Brain Tissue (Rabbit) When Tested Against Normal Plasmas.

Thromboplastin preparation from Rabbit No.	Prothrombin time (in sec)									
	Human plasma					Rabbit plasma				
	N.*	1†	2†	6†	8†	N.	1	2	6	8
CaCl ₂ concentrations										
.02 Molar	12	14	12½	13½	14	6	7	10½	9	9
.01 "	11½	12½	12½	12½	13½	6	7½	11	9	9
.005 "	11½	12½	12½	12	14	6	8	11	9½	9½
.0025 "	11	13½	13½	11½	15	5½	9	11	10	10½
.00125 "	14½	19½	20	16	22½	6	13	12	12	12½
.0006 "	26	72	39	48	43	8½	20	24	19	18
.0005 "	42	115	69	127	75	10½	28	31	22	27

* N.: Normal control.

Prothrombin time at time of death
using 0.01 M CaCl₂
28.5 sec.† Rabbit 1: Killed on 4th day (dicumarol by stomach tube)
" 2: " " 7th " "
" 6: " " 7th " "
" 8: " " 7th " " intravenously

to vascular damage.² Moreover, other factors of the normal mechanism of coagulation have been found to be affected by dicumarol. Adhesiveness of the platelets is decreased^{3,4} and the minimum requirement of calcium for optimum prothrombin time is increased^{5,6} during the administration of the drug.

Evidence has been obtained which indicates that dicumarol causes changes in the thromboplastic activity of tissues. Dyckerhoff⁷ claims that dicumarol inhibits the first phase of coagulation by depressing the activity of thrombokinase. Recently Bose⁸ has shown that thromboplastin prepared from desiccated brain of rabbits which have been fed 10 mg of dicumarol for 3 days is less active than that prepared from normal brains when tested on normal rabbit plasma using the prothrombin time technic.

In the present report, thromboplastic activity of rabbit brain obtained from animals severely poisoned by dicumarol has been studied by the prothrombin time method of Quick, but using a standard series of dilutions of CaCl₂. Plasma from men and from normal and dicumarol-treated dogs and rabbits was employed.

Experimental. Ten full grown male rabbits in good state of nutrition were given dicumarol [3,3'-methylenebis(4-hydroxycoumarin)] until the prothrombin level was so depressed that they either died from hemorrhage or their prothrombin time and coagulation time were so delayed that neither one could any longer be determined satisfactorily. The drug was administered in doses of 5 mg per kg of body weight per day. In some animals the drug was administered orally as a gum acacia suspension by stom-

² Bingham, J. B., Meyer, O. O., and Pohle, F. J., *Am. J. Med. Sc.*, 1941, **202**, 563.

³ Spooner, M., and Meyer, O. O., *Am. J. Physiol.*, 1944, **142**, 299.

⁴ Wright, H. P., *J. Path. Bact.*, 1945, **57**, 382.

⁵ Jaques, L. B., and Dunlop, A. P., *Am. J. Physiol.*, 1945, **143**, 355.

⁶ Quick, A. J., *Am. J. Physiol.*, 1947, **148**, 211.

⁷ Dyckerhoff, H., *Bioch. Z.*, 1944, **316**, 397, (*Chem. Abstracts*, 1945, p. 551).

⁸ Bose, A. N., *Ann. Biochem. Exp. Med.*, 1945, **5**, 105.

ach tube; in others intravenously as the sodium salt and in a third group both the oral and the intravenous routes were used. Thromboplastin was prepared by the acetone dehydration procedure of Quick.⁹ One-tenth of 0.1 M. sodium oxalate per rabbit brain was added before triturating with acetone. By this means any calcium in the brain tissue was removed, since no detectable amount of this element was found by ordinarily chemical methods in 0.2 g of the dried rabbit brain powder. The prothrombin time of oxalated human, dog and rabbit plasma was determined by the procedure of Quick. A standard series of dilutions of CaCl_2 solutions was used since the lower concentrations have been shown to be more sensitive to minimum changes of the prothrombin time.

Results and Discussion. It is evident from the results in Table I that the thromboplastin prepared from the brains of rabbits poisoned with dicumarol was less active than that obtained from normal animals. With dogs' and rabbits' plasmas this difference is obtained with the various dilutions of calcium used but becomes more accentuated in the lower concentrations. With human plasma this difference is obtained only in the lower concentrations.

In comparing the thromboplastin from dicumarolized rabbits with that of normal

animals on rabbit and dog plasmas in which the prothrombin was depressed by dicumarol, the difference in activity was found to have a wider spread (Table II) but followed essentially the pattern of normal oxalated plasma in regard to the influence of calcium. Occasionally when the prothrombin is presumably normal as measured with fully active thromboplastin, a distinct delay is brought out by dicumarol thromboplastin as shown in the case of dog No. 2 on the 9th day after a single dose of dicumarol intravenously.

It is somewhat surprising that dicumarol should decrease the activity of the thromboplastin in brain tissue and this naturally leads to the question, whether the thromboplastin occurring in blood is similarly affected. If this be the case, then the delayed coagulability due to dicumarol would have a 3-fold cause: decrease of prothrombin level, inadequate calcium for optimum prothrombin activity and less active thromboplastin.

What importance the latter factor has is difficult to evaluate especially since the diminution in thromboplastic activity is not particularly marked. In view of the fact that the nature of thromboplastin is poorly understood and the action of dicumarol is not completely known, it would be futile to attempt to offer any possible explanation for the results presented in this paper.

⁹ Quick, A. J., *Science*, 1940, **92**, 113.

15695

Grisein, a New Antibiotic Produced by a Strain of *Streptomyces griseus*.^{*†}

DONALD M. REYNOLDS, ALBERT SCHATZ, AND SELMAN A. WAKSMAN.

From the Department of Microbiology, New Jersey Agricultural Experiment Station, Rutgers University.

Streptomyces griseus represents a large and heterogeneous group of actinomycetes, widely distributed in nature. Cultures belonging to

this species or species-group have been isolated from a great variety of substrates, largely soils, peats, composts and animal contents. Since the first report¹ on the production of streptomycin by 2 cultures of *S. griseus* obtained from 2 different substrates,

^{*} Journal Series Paper, New Jersey Agricultural Experiment Station, Rutgers University. Department of Microbiology.

[†] Partly supported by a grant made by the Commonwealth Fund of New York.

¹ Schatz, A., Bugie, E., and Waksman, S. A., *Proc. Soc. EXP. BIOL. AND MED.*, 1944, **55**, 66.

TABLE I.
Spectrum of Cultures of *S. griseus* Grown in
Nutrient and Corn Steep Broths.
Dilution units per 1 milliliter.

	Nutrient broth	Corn steep broth
<i>Escherichia coli</i>	250	30
<i>Aerobacter aerogenes</i>	0	0
<i>Serratia marcescens</i>	250	50
<i>Pseudomonas aeruginosa</i>	5	0
" <i>fluorescens</i>	0	0
<i>Proteus vulgaris</i>	0	0
<i>Mycobacterium phlei</i>	0	0
<i>Bacillus subtilis</i>	200	30
" <i>mycoides</i>	0	0
" <i>megatherium</i>	100	100
<i>Staphylococcus aureus</i>	200	200
<i>Sarcina lutea</i>	0	0

nearly 100 cultures of this organism or closely related forms have been isolated.² However, very few of these were found to be capable of producing streptomycin. Actually, one other culture among the many isolated in our laboratory had the capacity of forming authentic streptomycin.³ Two other streptomycin-producing strains were reported from another laboratory.⁴

Morphologically, *S. griseus* is characterized by the formation of clusters or tufts of sporulating hyphae in the aerial mycelium. The most characteristic cultural property is the production on suitable media of an abundant, powdery, greyish-green aerial mycelium. No distinct chromogenic (dark brown to black) pigment is produced on organic media; occasionally a greenish to light brownish pigment is observed just below the vegetative growth. The typical cultures may give rise to variants which differ from the original organism both in their morphology and in their physiology. Two variants have already been isolated, one of which is devoid of aerial mycelium⁵ and the other showing certain other cultural characteristics which are distinct from those of the mother culture; both variants are unable to produce streptomycin. The ability of other variants to produce streptomycin

may be greatly reduced.

For the isolation of *S. griseus* from natural substrates, media enriched with streptomycin have been utilized.³ Such media prevent the development of most of the bacteria and the great majority of actinomycetes. Among a large number of cultures isolated by this method, one was found to form an antibiotic which at first appeared to be streptomycin-like in nature. This culture (G-25) was isolated from a sample of Huleh peat, obtained from Palestine. The antibiotic was produced on a variety of media, of which peptone-meat extract and starch-tryptone had the highest activity. Culture filtrates of the organism were found to inhibit the growth of various Gram-negative bacteria, notably *Escherichia coli*, as well as certain Gram-positive bacteria, such as *Bacillus subtilis* and *Staphylococcus aureus*.

In spite of certain apparent similarities of this antibiotic to streptomycin and to streptothricin, 4 important differences were noted, suggesting that one is dealing here with a type of substance distinct from the others: 1. The bacteriostatic spectrum of the new agent was much narrower than that of either streptomycin or streptothricin. A typical culture filtrate of G-25 had a titer of 300 *E. coli* and 300 *B. subtilis* dilution units, but,

TABLE II.
Bacteriostatic Spectra of Grisein and Streptomycin.
Units per gram of crude preparations.

	Grisein × 1,000	Streptomycin × 1,000
<i>Escherichia coli</i> W*	25	25
<i>Serratia marcescens</i>	10	25
<i>Proteus vulgaris</i>	<.1	10
<i>Pseudomonas fluorescens</i>	3	2
<i>Ps. aeruginosa</i>	<.1	1
<i>Aerobacter aerogenes</i>	<.1	10
<i>Salmonella schottmülleri</i>	10	15
" <i>acutryke</i>	<.1	3
<i>Enterobella typhi</i>	<.1	25
<i>Shigella</i> sp.	30	25
<i>Klebsiella pneumoniae</i>	5	25
<i>Mycobacterium phlei</i>	<.1	100
<i>Bacillus subtilis</i>	10 to 30	125
" <i>megatherium</i>	10 to 20	100
" <i>mycoides</i>	<.1	20
" <i>ecreus</i>	<.1	30
<i>Staphylococcus aureus</i>	30 to 100	15
<i>S. lutea</i>	0.5	100
<i>Micrococcus lysodeikticus</i>	200 to 300	150

* Considerable variation in sensitivity of different strains has been observed.

² Waksman, S. A., Schatz, A., and Reynolds, D. M., *Ann. N. Y. Acad. Sci.*, 1946, **48**, 73.

³ Waksman, S. A., Reilly, H. C., and Johnstone, D. B., *J. Bact.*, 1946, **52**, 393.

⁴ Carvajal, F., *Mycologia*, 1946, **38**, 596.

⁵ Waksman, S. A., Reilly, H. C., and Schatz, A., *Proc. Nat. Acad. Sci.*, 1945, **31**, 157.

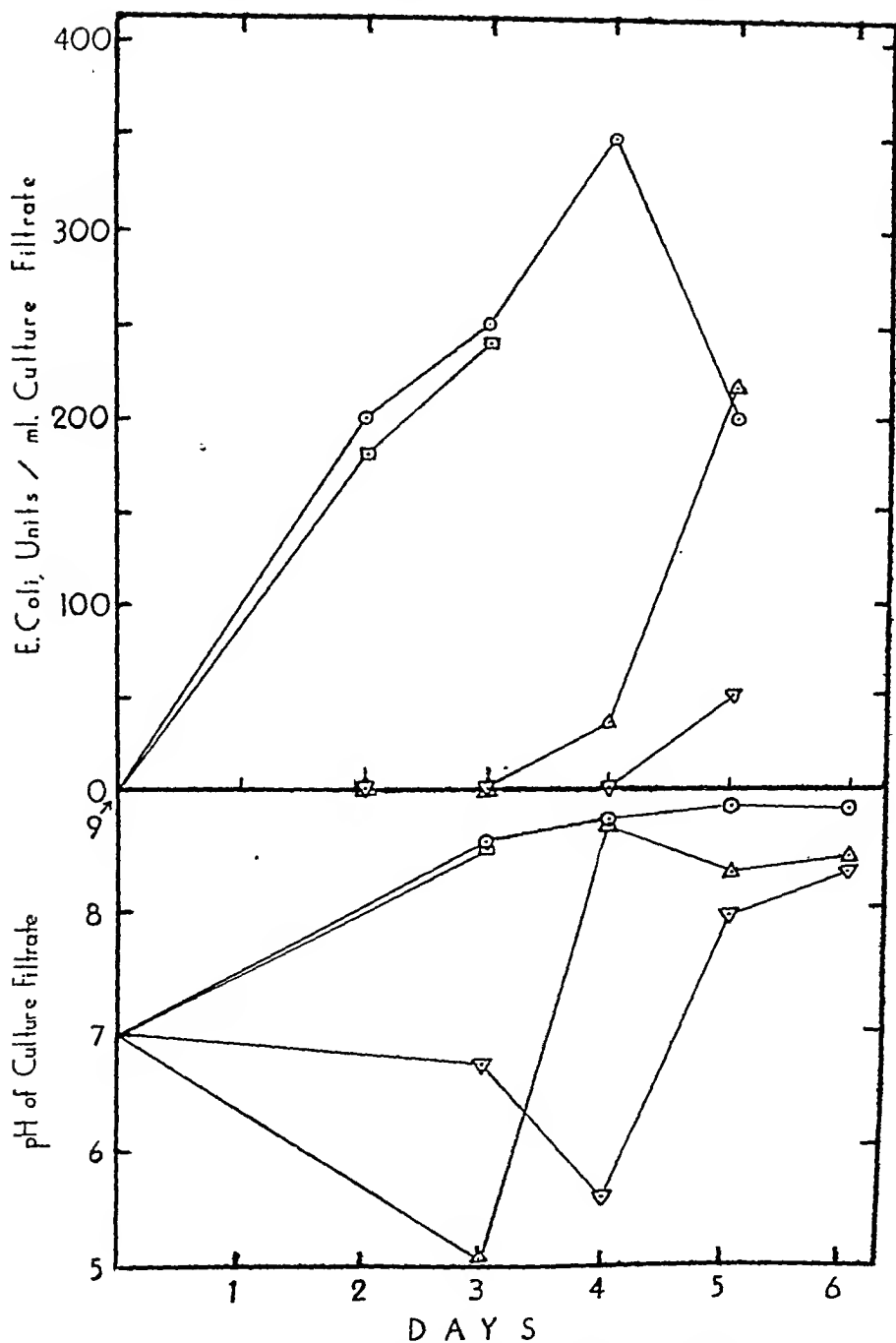


FIG. 1.

The Effect of Various Supplements on the Production of Grisein.

○ Basal medium—5 g peptone, 5 g NaCl, 10 ml neutralized corn steep liquor per liter.
 Supplements added—10 g/lit.; △, glucose; inverted △, glycerol; □, lactose.

unlike streptomycin, had no activity against either *B. mycoides* or *Acrobacter acrogeus*. Likewise, it had no activity against fungi, a characteristic which distinguishes it from streptothricin. 2. The chemical nature of the antibiotic produced by G-25 is different from that of the other 2 antibiotics, since, while readily adsorbed on charcoal, it could not be removed with acid alcohol. 3. Glucose did not bring about the inactivation of this antibiotic, though it usually inactivates both streptothricin and streptomycin. 4. Streptomycin-resistant strains of *E. coli* remained sensitive to this antibiotic.

Culture G-25 was grown both in stationary and in submerged cultures. The submerged culture underwent rapid, even if incomplete, lysis in 3 to 5 days. Media containing meat extract or corn steep, but free from glucose, gave the best activity. The bacteriostatic spectrum of a typical culture filtrate of this organism is brought out in Table I. The agar streak method⁶ was used. The results point to a very narrow antibiotic spectrum, which is not limited either to the Gram-positive or to the Gram-negative bacteria (Table II).

When the culture filtrate was treated with activated charcoal (5 g per liter), all the antibiotic was removed. On treatment of the charcoal with 9% ethanol, about half of the total activity was recovered. The alcohol eluate was concentrated to a syrup *in vacuo*, and absolute methanol was added until a precipitate began to form. The final preparation was precipitated with acetone, washed with ether, and desiccated. A yield of 400-600 mg was obtained per liter of culture. This preparation showed an activity of about 15,000-30,000 *E. coli* dilution units per gram. More active preparations have often been obtained, ranging up to 100,000 *E. coli* units per gram. Unfortunately, only a small part, usually about 20%, of the total active substance present in the culture was thus recovered. The antibiotic spectrum of the isolated fraction was, however, exactly the same as that of the crude culture filtrate, thus pointing to the identity of the substance

in the medium and of the isolated preparation.

It is proposed to designate this antibiotic substance as *grisein*. It is insoluble in ether, chloroform, absolute acetone, absolute ethanol, or benzene. It is slightly soluble in ordinary acetone and in 95% ethanol. It is readily soluble in water. It is heat-stable, since heating for 10 minutes at 100°C does not reduce greatly its activity. It is not affected if kept for short periods at pH levels ranging from 4.0 to 10.5.

The antibiotic activity of grisein can be measured by the dilution agar-streak and cup assay methods. With the cup method, *B. subtilis*, *S. aureus* and *E. coli* can be used as test organisms; the zone of inhibition, for the first organism, is usually narrower than that for comparable concentrations of either streptomycin or streptothricin, but it is wider and more distinct for the second and third organisms. The broth dilution method cannot be used very effectively for the study of grisein, since there are always a few cells in the culture of the test bacterium that are originally resistant or become readily resistant to this antibiotic; these cells begin to multiply rapidly, making impossible accurate readings by the dilution method. The rapid development of resistant strains from originally sensitive cultures makes studies of the bactericidal action of grisein upon bacteria in broth cultures rather difficult.

The presence of glucose or glycerol in the culture medium for the production of grisein results in very low yields of this antibiotic due to a lowering of the pH. However, as soon as the pH begins to rise, the antibiotic is formed, as shown in Fig. 1. Lactose, however, has no such effect.

Samples of crude grisein preparations were tested in mice infected with *Salmonella schottmülleri* and *S. aureus*. A single dose of 800 units (dilution units against *E. coli*) per mouse, administered subcutaneously immediately after infection, protected 100% of the animals infected with the first, and 1,600 units/mouse gave complete protection against the second. The antibiotic showed only little toxicity to experimental animals and was excreted readily and in an active state in the urine. Because of its low toxicity, its activity *in vivo* and also its activity upon

⁶ Waksmun, S. A., and Reilly, H. C., *Anal. Ed., Ind. Eng. Chem.*, 1945, **17**, 556.

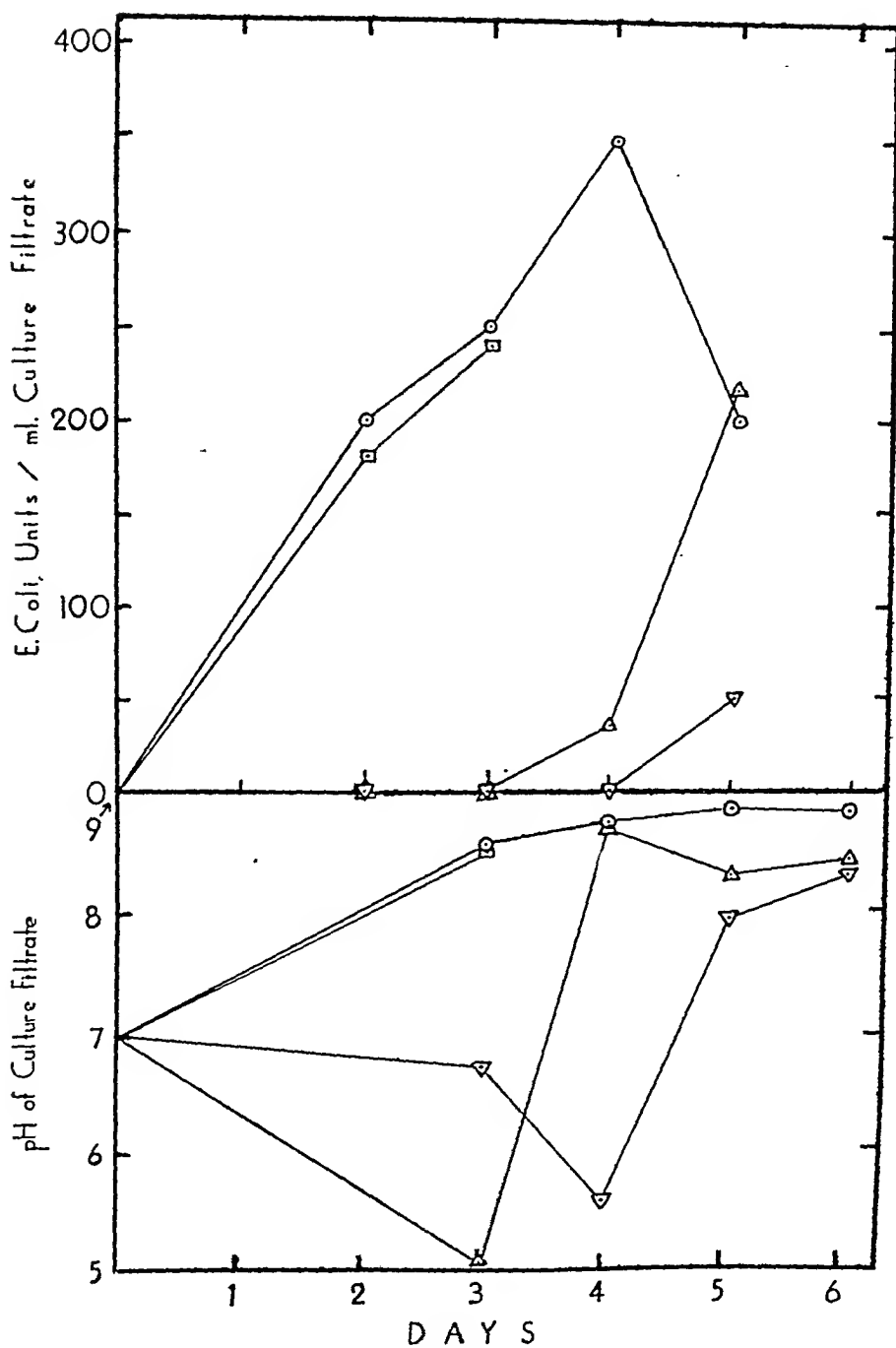


FIG. 1.

The Effect of Various Supplements on the Production of Grisein.

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 Supplements added—10 g/lit.; △, glucose; inverted △, glycerol; □, lactose.

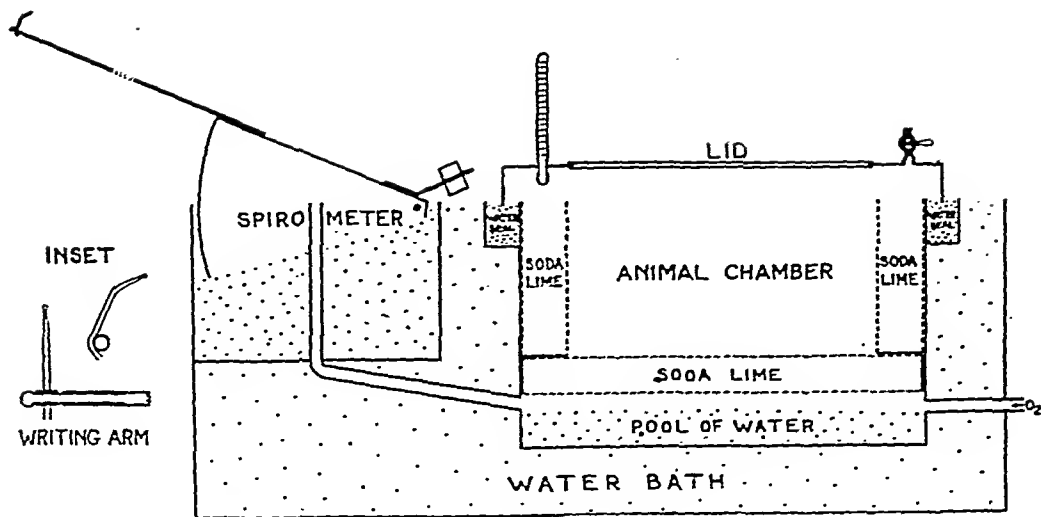


FIG. 1.

Basal Metabolism Machine. Longitudinal section. About $\frac{1}{4}$ scale. Heating element, thermoregulator, and water stirrer not shown. Inset: writing arm, side view as inserted into writing lever, end view of writing arm and collar without the pin which inserts into writing lever.

tion of water. The thermometer¹ and a stopcock, to allow free movement of air into the system whenever the apparatus is opened, are fitted into the lid. The chamber is rendered airtight by a water-filled moat into which the overhanging rim of the lid dips.

The rat is placed into a wire mesh cage, somewhat smaller than the space available, and this is introduced in turn into the animal chamber, where it is flanked fore and aft by soda lime boxes made of copper window screening. The inner cage rests on a false-bottom floor, formed by a third soda lime container, which is itself elevated about an inch above the true floor of the animal chamber in order to provide space for a pool of water below the CO_2 absorbent.

Koehler² points out the advisability of maintaining a relatively high humidity within the chamber, partly to keep the soda lime at maximum absorptive efficiency and partly to induce the animal to relax. He

considers, however, that a humidity above 75% causes restlessness. The present author, on the other hand, observed that the soporific influence varies directly with the vapor tension and that the 99% humidity provided at 28°C by a pool of water is most effective, at least in the rat. Under that regime, the animal almost immediately composes itself for sleep, sometimes on the first trial, usually after 3 or 4.

The spirometer bell is made of thin-walled brush copper, which is far less vulnerable than aluminum to attack by soda lime. It is a Krogh type spirometer, pivoting on jewel bearings and thus providing freedom of movement without the lateral shifting encountered in the ordinary cylindrical spirometer. The bell is counterweighted in such manner that, with the system open to the outside, it descends slowly and without acceleration. Its dimensions are $5'' \times 2\frac{1}{2}'' \times \frac{3}{4}-3\frac{1}{2}''$, giving a volume of about 300 cc, which is well above the requirement of even a hyperthyroid rat. Customarily, however, the spirometer is refilled during the rest period about 10 minutes before the test run; this is done from an ordinary vitalometer in order to avoid disturbance of the rat by the

¹ The thermometer manufactured by the Warren E. Collins Co., Boston, for use in the Benedict Roth Metabolism Machine has a brass jacket which can be screwed and cemented in place.

² Koehler, A. E., *J. Biol. Chem.*, 1932, 95, 67.

streptomycin-resistant strains point to the possibility of utilizing grisein for the control of infections caused by bacteria resistant to other antibiotics. However, its narrow antibiotic spectrum would limit its practical application.

Summary. *Streptomyces griseus* is widely distributed in soils, peats and in composts. Only very few strains of this organism are capable of producing streptomycin. Most of the strains produce no antibiotic at all, whereas certain strains produce antibiotics that are distinctly different from streptomycin. One such new antibiotic was isolated and designated as grisein.

Grisein is active against certain Gram-positive and Gram-negative bacteria. Its antibacterial spectrum is much more limited than

that of either streptomycin or streptothricin. Cultures of bacteria that are made resistant, by serial passage, to streptomycin still remain sensitive to grisein. Cultures of bacteria originally sensitive to grisein give rise easily to strains resistant to this antibiotic.

Grisein shows a rather low toxicity to experimental animals and is rapidly excreted in the urine. It was found capable of protecting experimental animals against infections with *S. schottmülleri* and *S. aureus*.[†]

[†] The authors are indebted to Mr. Otto Graessle, of the Merck Institute, for testing the animal toxicity of grisein, and to Dr. H. Boyd Woodruff, of the Merck Laboratories, for checking the sensitivity of streptomycin-resistant strains of different bacteria.

15696

Determination of Oxygen Consumption in the Albino Rat.*

LOUIS E. MOSES. (Introduced by F. E. Emery.)

From the Department of Physiology and Pharmacology, University of Arkansas School of Medicine, Little Rock.

Technical aspects of the measurement of gaseous metabolism in the albino rat would appear, from a cursory survey of the literature, to be almost a closed chapter in biologic technology. Yet the determinations, as executed, have proved inaccurate, inconsistent, and time-consuming. The method to be described below appears to offer several distinct advances over those currently in use.

The machine devised for these studies is of the closed-circuit type, measuring only oxygen consumption. Its major structural features are illustrated in Fig. 1.

Animal chamber, spirometer box, and water bath are all constructed of heavy sheet brass, which is adequate for the rapid equalization of temperature throughout the system. The temperature is maintained within narrow limits by means of a tubular model heating

element controlled through a suitable relay by a thermoregulator immersed in the water bath. The water is kept circulating by a noiseless electric stirrer. Sensitivity of the thermoregulator is assayed by a blank test, measuring the variations in volume of the inclosed oxygen that are induced by the automatic activation and deactivation of the heating element; such variations have not exceeded 0.5 cc. The temperature within the animal chamber is maintained at 28°C, stated by Benedict and MacLeod¹ to be the temperature of thermic neutrality for the albino rat.

Dimensions of the animal chamber are 9" x 6" x 5½". Its lid is provided with a large window, composed of 2 panes of glass separated by an air space to limit condensa-

* Paper No. 831, Research Series, the University of Arkansas.

¹ Benedict, F. G., and MacLeod, G., *J. Nutrition*, 1929, 1, 367.

in convenient form by transfer onto a plate of window glass of the record made by withdrawal of successive 5 cc volumes of air from the chamber into an accurate burette. The rulings are scratched on this glass plate with a diamond point and are then filled with colored wax; the transparent rule thus produced can be placed on each tracing and used to read off the oxygen consumption values accurately and quickly. Simultaneous ordinates for the signal magnet time record are transcribed onto the oxygen line of each tracing by manual operation of the drum and writing lever, after the test run.

In practice, then, the fasted rat is placed into the small wire mesh cage referred to above, and this is inserted into the animal chamber of the basal metabolism machine. Oxygen is admitted and allowed to flow out of the open stopcock and to bubble out of the spirometer until all air in the system is replaced by oxygen. Then the chamber is closed and at least 30 minutes are allowed for the animal to fall asleep and for the system to reach equilibrium. A 30-minute tracing is made and the best 5-minute period noted, along with an estimate of activity as determined from the smoothness of the tracing.

When the oxygen line indicates by an absence of any waves that the rat has been sleeping soundly throughout the recording period, all of the 5-minute readings give practically identical rates of oxygen consumption. Any movement which does occur elevates the succeeding readings in direct proportion to the intensity and duration of the activity:

even a mere shift in position by a visibly sleeping animal increases the oxygen consumption for at least the next 5-minute period.

The graph of Fig. 2 shows the distribution of values, expressed as cc/kg/hr, according to age, in 172 determinations on 42 adult male rats. Only those observations made on sleeping animals are included, and all rats were housed in a constant temperature room at 26°C. Averages for the individual age groups show, with other observers, a progressive decline with age, reaching what appears to be a plateau at about 6 months. The mean oxygen consumption of the 133 determinations made in the 6-9-month age range is 692 cc/kg/hr \pm 3.0 (P. E.), which may be taken as the basal metabolic rate obtained in "metabolic maturity." This figure is equivalent to 635 Cal/m²/24 hr, using the formula, $S. A. = 9.1 \times W^{2/3}$, for calculation of surface area.

Summary. 1. A method is described for determination of oxygen consumption in the rat. Its major advances are: (a) Humidity control, inducing sleep. (b) Sensitive recording method, which shows body movements on the oxygen line and makes feasible readings covering 5-minute periods.

2. Values obtained in 172 determinations on 42 male rats show a decline from 771 cc/kg/hr at age 4 months and 752 cc/kg/hr at 5 months to a plateau at 6 months and maintained to at least 9 months. The average for 133 determinations on rats of 6-9 months is 692 cc/kg/hr \pm 3.0 (P. E.), or 635 Cal/m²/24 hr.

15697

The Susceptibility to Furacin of Bacterial Strains Resistant to Sulfonamides or Antibiotics.*

MORRIS N. GREEN AND STUART MUDD.

From the Department of Bacteriology, School of Medicine, University of Pennsylvania, Philadelphia, Pa.

Furacin or 5-nitro-2-furaldehyde semicarbazone has been found to be both bac-

tericidal and bacteriostatic for a large group of Gram-positive and Gram-negative bac-

* This work has been aided by a grant from the

Eaton Laboratories, Inc., Norwich, N.Y.

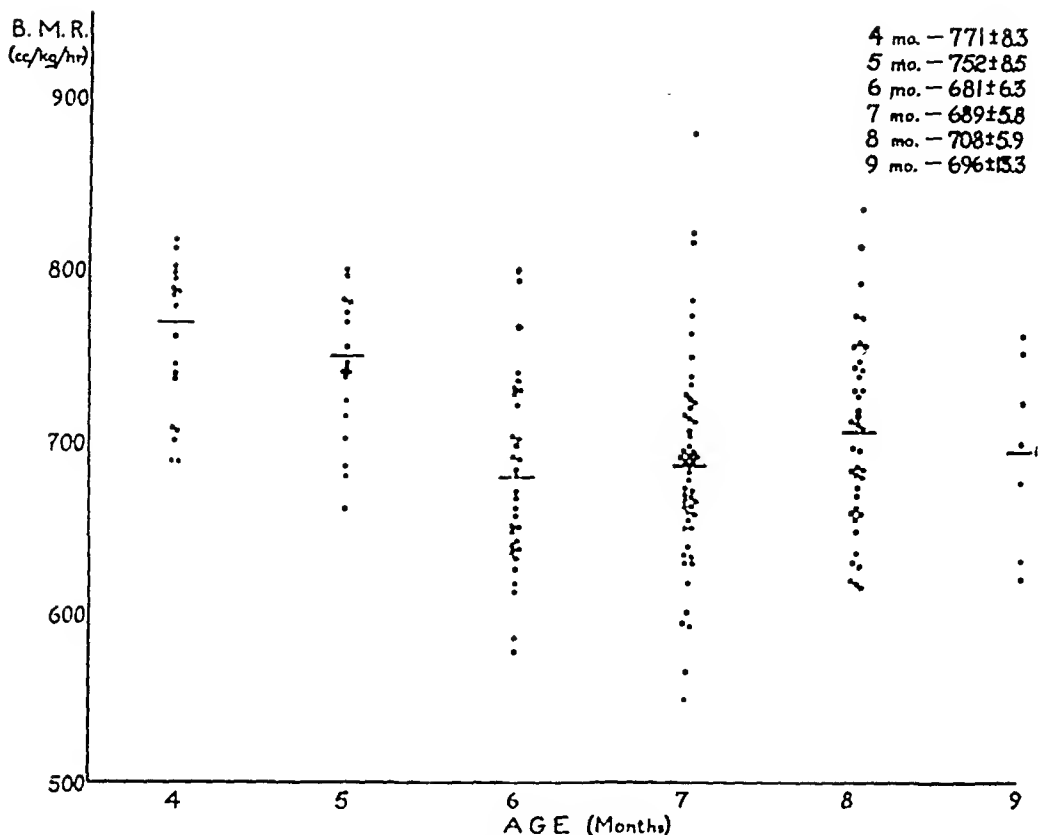


Fig. 2.

Relation of Oxygen Consumption to Age in Male Rats. Mean and probable error for each age level shown at upper right corner.

hiss of gas as it comes from a compressed oxygen tank.

At the tip of the tubular stainless steel writing lever is attached a free-swinging writing arm made of capillary glass tubing (see inset, Fig. 1). Such a writing point exerts a constant light pressure, determined solely by its own weight, against the kymograph drum. Fire-polishing the tip converts it into a smooth ball of low friction.

Use of the spirometer and writing point described above leads to a high degree of sensitivity, so much so that the slightest movement of the rat communicates itself as a gross hump to the otherwise smooth kymogram. Such precision affords an integral record of activity on the oxygen line; it also makes possible the measurement of oxygen consumption over so short an interval as 5

minutes. Such short periods were found to give lower and more consistent readings than the much more prolonged runs usually considered necessary.

Inscription of the tracing in this manner, however, introduces complications in measurement, since it brings about an interplay between 3 arcs, described by the drum's periphery, the spirometer bell, and the free-swinging writing arm. This requires that steps be taken to insure a constant, exactly reproducible spatial relationship between the kymograph and the basal metabolism machine, and that a measuring rule be made for the individual machine. The former control is easily made by fastening the machine and the kymograph to wooden bases so shaped that they fit together in only one position. The measuring rule is constructed

penicillin-resistant and susceptible strains of *S. aureus* were obtained. These strains were isolated before and after penicillin therapy.

The following procedure was used in testing the cross resistance of these strains:

All reagents were diluted either in casein hydrolysate or extract broth. The standard test inoculum for all strains was 200,000 bacteria per ml. The strains were checked for their resistance or susceptibility to penicillin, streptomycin or sulfonamides, respectively. All of the strains were tested against various dilutions of furacin to determine whether or not there was any difference in susceptibility to this drug of the resistant and susceptible strains. Daily visual observations of growth were made for 4 days and the results recorded as negative (no visible growth), doubtful, 1+, 2+, 3+ or 4+ growth. Casein hydrolysate medium was used for testing the penicillin and sulfonamide strains, extract broth for testing the streptomycin strains.

Results. In Table I representative results are given for each of the drugs tested. Not all of the results are given. Those omitted were essentially similar in character to those given in Table I. Except for some of the penicillin strains, a fairly high order of resistance to the various drugs tested was obtained. None of the paired organisms resistant to sulfathiazole, streptomycin or penicillin showed any change in resistance to furacin.

In most cases identical results with furacin were obtained with both the resistant and susceptible members of a pair of organisms. In some cases at the end of 48, 72, or 96 hours, differences were observed in the highest dilution inhibiting growth: that is, one member of a pair would show growth only at the next lower dilution. This reaction was just as likely to occur in the resistant as the susceptible strain of a pair of organisms.

Several experiments were tried with pairs of pneumococci^{1b} resistant to sulfathiazole, acriflavine, atabrine and optochin. However, these strains grew at the highest possible concentration of furacin (1:5000), both under aerobic and anaerobic conditions, with minute inocula. The relative resistance to furacin, therefore, could not be ascertained.

Summary and Conclusions. Several Gram-negative and Gram-positive organisms resistant to sulfonamides, streptomycin and penicillin and the parent susceptible strains were tested in parallel for their susceptibility to furacin. No differences were observed between the resistant and susceptible strains in regard to their susceptibility to furacin. Resistance to penicillin, streptomycin or sulfathiazole does not result in any change to furacin resistance.

The authors wish to thank Dr. Morton Klein of this laboratory for suggestions and criticism during the course of the work, and Mr. Joseph Gots, also of this laboratory, for carrying out the experiments with pneumococci.

15698 P

The Corneal and Lenticular Changes Resulting from Amino Acid Deficiencies in the Rat.

V. P. SYDENSTRICKER, HENRY L. SCHMIDT, JR., AND W. KNOWLTON HALL.

From the Departments of Medicine and Biochemistry, University of Georgia School of Medicine, Augusta, Ga.

Corneal vascularization is a tissue change which may result from certain nutritional deficiencies.¹ Totter and Day² observed that

¹ Dann, W. J., and Darby, W. J., *Physiol. Rev.* 1945, 25, 326.

this change resulted in the rat from deficiencies of tryptophane or lysine. Maun, Cabill and Davis found that corneal vascularization

² Totter, J. R., and Day, P. L., *J. Nutrition*, 1942, 24, 159.

terial.¹ It has found practical application in the local treatment of infected areas when combined with carbowaxes and propylene glycol as a washable base.² In connection with its clinical application, it is of interest to determine whether or not organisms resistant to sulfonamides, penicillin or streptomycin are also resistant to furacin. The experiments reported in this paper have shown that pairs of strains respectively resistant and susceptible to sulfonamides, penicillin or streptomycin do not differ in their susceptibility to furacin.

Methods and Materials. Five pairs of coagulase-positive strains of *Staphylococcus aureus* were obtained—one member of each pair resistant and the other susceptible to sulfonamides. The resistant strains had been derived from the parent strain by continuous transfer in increasing sulfonamide concentrations. For streptomycin similar pairs were obtained comprising *Shigella paradyseuteriae*, *Escherichia coli*, *Proteus vulgaris*, *Streptococcus viridans*, and *Staphylococcus albus*. The dysentery strains were obtained through the courtesy of Dr. Morton Klein of this laboratory. The resistant strain was obtained by culturing the organism in the presence of increasing concentrations of streptomycin. The others were obtained through the courtesy of Dr. C. W. Bugge of Wayne University School of Medicine. They were isolated from a patient who suffered from an abdominal gun shot wound and was treated with 4 g of streptomycin every 24 hours. The parent strains were isolated before therapy and the resistant ones at various times during therapy. Through the courtesy of Dr. John E. Blair, Hospital for Joint Diseases, New York, 3 pairs of coagulase-positive

¹ (a) Dodd, M. C., and Stillman, W. B., *J. Pharm. Exp. Therap.*, 1944, **82**, 11; (b) Dodd, M. C., *J. Pharm. Exp. Therap.*, 1946, **86**, 311; (c) Cramer, D. L., and Dodd, M. C., *J. Bact.*, 1946, **51**, 293.

² (a) Snyder, M. L., Kiehn, C. L., and Christopherson, J. W., *The Military Surgeon*, 1945, **97**, 380; (b) Dodd, M. C., Hartmann, F. W., and Ward, W. C., *Surgery, Gynecology, and Obstetrics*, 1946, **83**, 73; (c) Shipley, E. R., and Dodd, M. C., *Surgery, Gynecology, and Obstetrics*, in press; (d) Neter, E., and Lamberti, T. G., *Am. J. Surgery*, 1946, **72**, 246.

TABLE I.
Growth of Organisms Susceptible and Resistant to Various Chemotherapeutic Agents in Dilutions of Furacin.*

Chemotherapeutic agent	Inhibitory concentration	Organisms	Dilutions of Furacin										
			10,000	25,000	50,000	75,000	100,000	250,000	500,000	750,000	1,000,000	Control	
Sulfathiazole†	1/10,000	<i>S. aureus</i> 5A	—	—	—	—	—	—	—	—	—	—	4
"	1/1000	" 5B	—	—	—	—	—	—	—	—	—	—	4
Streptomycin	<16 γ /ml	<i>E. coli</i> 135A	—	—	—	—	—	—	—	—	—	—	4
"	>5000 γ /ml	" 167A	—	—	—	—	—	—	—	—	—	—	4
"	>32 γ /ml	<i>P. vulgaris</i> 196B	—	—	—	—	—	—	—	—	—	—	4
"	>5000 γ /ml	" 198B	—	—	—	—	—	—	—	—	—	—	4
"	>200 γ /ml	<i>S. viridans</i> 135B	—	—	—	—	—	—	—	—	—	—	4
"	>5000 γ /ml	" 179	—	—	—	—	—	—	—	—	—	—	4
"	<1 γ /ml	<i>S. albus</i> 146B	—	—	—	—	—	—	—	—	—	—	4
"	150 γ /ml	" 241B	—	—	—	—	—	—	—	—	—	—	4
"	25 γ /ml	<i>S. paradyseuteriae</i> (Flexner 15)	—	—	—	—	—	—	—	—	—	—	4
"	>1000 γ /ml	" "	—	—	—	—	—	—	—	—	—	—	4
Penicillin‡	0.1 unit/ml	<i>S. aureus</i> 726	—	—	—	—	—	—	—	—	—	—	4
"	>4 units/ml	" 726R	—	—	—	—	—	—	—	—	—	—	4

* 48-hour readings are given as representative of the results obtained.

† No visible growth.

‡ 5 pairs tested.

§ Actual dilutions used were 200,000 and 400,000 instead of 20,000 and 500,000.

of the deficient, nor those of the control animals showed alterations."

From the work reported or cited here it appears that corneal vascularization is a reaction which results in the rat from a deficiency of any of the indispensable amino acids or from protein deprivation.

To date, 4 of the 7 rats on the histidine-deficient diet have developed bilateral cataracts of varying size. It is to be noted that these 4 rats are all from different litters and that none of the rats on the other diets used in this study have shown any lenticular changes which could be seen with the biomicroscope.

Summary. In the rat, deficiencies of leucine, isoleucine, phenylalanine, histidine, threonine, valine, or of arginine, resulted in corneal vascularization. Four of 7 rats on a histidine-deficient diet developed lenticular opacities.

This study was aided by grants from the John and Mary R. Markle Foundation and the U. S. Public Health Service. Some of the amino acids used in this work were donated by Winthrop Chemical Company and by the Corn Products Refining Company.

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Chemical Changes in the Developing Turtle Embryo.

THEODOR VON BRAND AND W. GARDNER LYNN.

From the Department of Biology, The Catholic University of America, Washington, D.C.

It has been found in previous experiments¹ that the embryo of the snapping turtle (*Chelydra serpentina*) consumes fat in the amount of 61 mg per individual during the last 57 days of its development. Data obtained on the loss of dry substance during the same period, in conjunction with figures for fat loss and oxygen consumption, led to the assumption that relatively large amounts of protein were probably metabolized also. In the present study determinations of protein, carbohydrate, inorganic substances, dry substance and fat were made on turtle eggs at the beginning of development and on newly hatched turtles. The eggs used were collected from the oviducts of 3 large snapping turtles on June 11, 1946 and each egg was weighed individually. Fifteen of the eggs (5 from each clutch) were opened at once and the egg contents (albumen and yolk) of all 15 were put into a single vessel and dried at 110°C to serve for the determination of the initial chemical composition. The other eggs were incubated at room temperature and when the young

turtles hatched (72-77 days) each was weighed and 15 specimens (5 from each clutch, as in the initial egg batch) were placed in a vessel and dried at 110°C. Eggs of as nearly as possible the same initial weights were used. The uniformity of the material is indicated by the fact that the average weight of the 15 eggs used for analysis of the chemical composition at the beginning of development was 11.13 ± 0.11 g (eggshells included) while the initial average weight of the 15 eggs which gave rise to the hatched turtles analyzed was 11.10 ± 0.09 g.

The 2 lots of dried material were each ground to a fine powder in a grinding mill and the following fractions were determined in aliquot parts by the methods specified: nitrogen according to Kjeldhal, using the customary factor of 6.25 to convert to protein; fat according to Kumagava-Suto;² total carbohydrate according to Dische and Popper³ and inorganic substances by inciner-

¹ Lynn, W. G., and von Brand, T., *Biol. Bull.*, 1945, **88**, 112.

² Kumagava, M., and Suto, K., *Bioch. Z.*, 1908, **8**, 212.

³ Dische, Z., and Popper, H., *Bioch. Z.*, 1926, **175**, 371.

appeared in deficiencies of leucine³ and of histidine⁴ but no histological changes in the cornea were observed when rats were made deficient in phenylalanine.⁵ Methionine⁶ or protein deprivation⁷ may similarly produce marked corneal changes, one of the most obvious of which is corneal vascularization. The suggestion has been made that corneal vascularization may be a reaction resulting from a deficiency of any of the indispensable amino acids or of protein.⁸ In the following report is an account of the corneal changes which we have observed to occur in deficiencies of certain of the indispensable amino acids.

Methods. Rats from a Wistar strain which were 25 or 26 days of age were placed in individual cages and given an experimental diet and water *ad libitum*. The control diets contained an amino acid mixture as used by Maun, Cahill and Davis⁹ in their control diet. This supplied approximately 3 times the amount required for normal growth of each of the indispensable amino acids. The remainder of our control diet contained in each 100 g: salt mixture¹⁰ 4 g, cod liver oil 2 g, cottonseed oil 3 g, choline chloride 0.2 g, riboflavin 1.6 mg, thiamin hydrochloride 0.4 mg, calcium pantothenate 2 mg, pyridoxin hydrochloride 0.4 mg and sufficient sucrose to make 100 g. Diets deficient in phenylalanine, leucine, isoleucine, arginine, histidine, threonine and valine were prepared by replacing the appropriate amino acid in the formula of the control diet with sucrose. Eight litters of 6 rats, one litter of 4. and

one of 5 rats were distributed among the diets so that there were 7 rats from different litters on each diet except for 8 on the threonine-deficient diet. Biomicroscopic examination of the rats' eyes were made 3 or 4 times weekly. One rat from each group where corneal vascularization occurred was changed to the control diet to induce regression of the vascularization. Further details of technics used here were described in an earlier paper.¹⁰ As before, we limit our use of the term corneal vascularization to the situation where there is an actual growth of capillaries in the cornea beyond the limits of normal variation.

Results. In all of the amino acid deficiencies investigated in this study some degree of corneal vascularization occurred. It was the most prompt and extreme in phenylalanine deficiency although the vascularization resulting from isoleucine and leucine deficiencies was nearly as extreme. Successively lesser degrees of corneal vascularization were observed in histidine, valine, threonine and least in arginine deficiencies. Of the 7 animals in each group on deficient diets the number which have showed corneal vascularization are respectively: phenylalanine 7, leucine 6, isoleucine 7, arginine 5, histidine 7, threonine 7 (of 8 rats), and valine 5. One of the control rats developed a few capillary loops into the edge of the cornea, beyond the usual limits of variation. Rats deficient in leucine, isoleucine, phenylalanine, histidine and threonine, when returned to the control diet to supply the lacking amino acid, underwent a prompt regression of vascularization until no corneal capillaries could be seen with the biomicroscope.

The observations described above as to the corneal vascularization occurring in leucine and histidine deficiencies are in accord with the findings of Maun, Cahill and Davis^{1,2} in these deficiencies. Our findings in phenylalanine deficiency are in contradiction to those of Maun, Cahill and Davis,³ since they reported that "neither the eyes

³ Maun, M. E., Cahill, W. M., and Davis, R. M., *Arch. Path.*, 1945, **40**, 173.

⁴ Maun, M. E., Cahill, W. M., and Davis, R. M., *Arch. Path.*, 1946, **41**, 25.

⁵ Maun, M. E., Cahill, W. M., and Davis, R. M., *Arch. Path.*, 1945, **39**, 294.

⁶ Berg, J. L., Pund, E. R., Sydenstricker, V. P., Hall, W. K., Bowles, L. L., and Hock, C. W., *J. Nutrition*, in press.

⁷ Hall, W. K., Sydenstricker, V. P., Hock, C. W., and Bowles, L. L., *J. Nutrition*, 1946, **32**, 509.

⁸ Sydenstricker, V. P., Hall, W. K., Hock, C. W., and Pund, E. R., *Science*, 1946, **103**, 194.

⁹ McKibben, J. M., Madden, R. J., Black, S., and Elvehjem, C. A., *Am. J. Physiol.*, 1939, **128**, 102.

¹⁰ Bowles, L. L., Allen, L., Sydenstricker, V. P., Hock, C. W., and Hall, W. K., *J. Nutrition*, 1946, **32**, 19.

TABLE I
Effect of Hemorrhage on the Absorption of Magnesium Sulfate.

Exp. No.	Magnesium absorption		Sulfate absorption	
	Control, %	Bled, %	Control, %	Bled, %
1.	—	21	—	11
2.	15	17	13	17
3.	24	17	24	18
4.	17	19	21	17
5.	5	26	10	28
6.	16	18	11	18
7.	10	17	9	17
8.	—	9	—	7
9.	18	—	11	—
10.	5	10	8	14
	—	—	—	—
Arg	14	17	13	16*

* None of the differences between the means are significant by Fisher's *t* test.

statistically significant.

It was thought worthwhile to ascertain whether anemic anoxia (hemorrhage) would influence the rate of absorption of magnesium sulfate from the small intestine. It is known that this salt, if absorbed in sufficient quantities, may be quite toxic, so the problem is of practical as well as of academic interest.

Methods. Paired dogs as nearly alike in weight and age as possible were fasted 24 hours. One served as a control and the other was subjected to hemorrhage. Ten experiments were performed on a total of 20 animals.

The experimental animal was etherized, the femoral artery cannulated and blood withdrawn equal to 3% of the body weight. The artery was then ligated and the incision closed. Four hours were allowed for recovery from the ether and adjustment of the circulatory mechanism. During this interim, water was allowed *ad libitum*.

The animal was then given sodium barbital intravenously (300 mg/kg). The small intestine was exposed by a mid-line incision and practically the entire ileum used for a loop. The length of the loop was made the same as that in the control animal. It was washed out with isotonic glucose solution and filled with an isotonic solution of magnesium sulfate (3.25% anhydrous). After 90 minutes the fluid was removed from the loop and carefully measured. It was digested

with concentrated HCl and the amount of sulfate determined gravimetrically by precipitation with barium chloride. The magnesium was determined by precipitation with 8-hydroxyquinoline.

The control animal was treated exactly as the experimental with the important exception, of course, that it was not bled.

Results. Table I shows the results obtained. There was no great difference of absorption of either magnesium or sulfate between the controls and those subjected to hemorrhage. The controls absorbed 13% of the sulfate and 14% of the magnesium, while the experimental animals absorbed 16% and 17% respectively. The differences in the means of the results were not statistically significant. There was a close parallelism between the absorption of magnesium and of sulfate. In all cases, save one, the same amount or more of fluid was recovered than was put into the intestinal loop. In one experimental animal 13 cc less was recovered than was put in. The greatest amount of secretion was 11 cc.

Discussion. The results clearly indicate that a pronounced hemorrhage does not make the intestinal epithelium more permeable to either the magnesium or the sulfate ion.

As pointed out previously¹ it is of distinct interest that an appreciable amount of magnesium sulfate is absorbed from the small intestine, even in a period of 90 minutes. This is not generally appreciated, although

TABLE I.
Chemical Changes in the Developing Embryo of the Snapping Turtle.

g	Egg	Hatched turtle	Difference
Avg fresh wt	9.89	8.01	-1.88
" dry "	2.07	1.68	-0.39
" protein content	1.15	0.93	-0.22
" fat "	0.52	0.30	-0.22
" carbohydrate content	0.14	0.16	+0.02
" inorganic "	0.17	0.19	+0.02

ation.

Our results are summarized in Table I. It is obvious that the main substances consumed were fat and protein. Quantitatively, identical amounts of both groups of substances disappeared, but the greater amount of energy was obviously derived from the fat combustion. It will be noted that the sum of protein, fat, carbohydrate and inorganic substances amounts to about 95% of the dry weight in both batches. The 5% which is unaccounted for may be due to errors in the protein and carbohydrate determinations. It is questionable whether the customary conversion factor of 6.25 used in the protein determination is exactly applicable to turtle protein and it may well be that the carbohydrates present do not give exactly the same color intensity with the indole reagent as does the glucose solution used for comparison. The quantitative balance is sufficiently close, however, to prove that the developing snapper embryo derives its en-

ergy from the combustion of fat and protein. Similar observations have thus far been reported for only one other chelonian, the marine turtle *Thalassochelys corticata*.^{4,5}

In our previous study¹ we found that a snapper egg consumes about 500 cc of oxygen during its development. The complete oxidation of 0.22 g of fat and 0.22 g of protein would require about 660 cc of oxygen. If some carbohydrate were synthesized from protein, the figure would be slightly lower, but would still be in excess of 500 cc. The discrepancy may be explained by the fact that the eggs used this year were somewhat heavier than those employed 2 years ago (11.1 g as compared with 9.9 g) and that their development required an average of 75 days as against 72 days.

⁴ Karashima, J., *J. Biochem. (Tokyo)*, 1929, 10, 375.

⁵ Nakamura, Y., *J. Biochem. (Tokyo)*, 1929, 10, 357.

15700

Effect of Anemic Anoxia on Absorption of Isotonic Magnesium Sulfate from the Small Intestine.*

E. J. VAN LIERE, D. W. NORTHUP, J. C. STICKNEY, AND R. E. RICHARD.

From the Department of Physiology, West Virginia University, Morgantown.

It was shown by Northup and Van Liere¹ that even severe degrees of anoxic anoxia

* Aided by a grant of the Ella Sachs Plotz Foundation.

¹ Northup, D. W., and Van Liere, E. J., *Arch. Internat. Pharmacol. et de Therap.*, 1939, 62, 175.

² Van Liere, E. J., Northup, D. W., and Sleeth, C. K., *Am. J. Physiol.*, 1938, 124, 102.

had no significant effect on absorption of magnesium sulfate from the small intestine of dogs. It had previously been shown,² however, that in anemic anoxia, produced by bleeding dogs 3.2% of their body weight, isotonic sodium chloride solution was absorbed from the small intestine more quickly than in control animals. The results were

The present paper describes a sensitive fluorimetric method for assay of the steroid estrogenic hormones on the basis of the fluorescence afforded when these substances are heated with phosphoric acid.

Method for estrone, estradiol and estriol in pure solutions. It has been known for some time that several natural estrogens—estrone, estradiol, estriol—produce on warming in concentrated sulfuric acid orange-colored solutions with green to blue fluorescence.¹¹⁻¹³ The fluorescence reaction has been employed widely as a qualitative test for estrogen in preparative work, and diagnostically as a qualitative test for pregnancy in the mare.^{14,15} As sulfuric acid produces color under the same conditions, and in some cases fluorescence with a large range of substances, it seems not to have been considered worthwhile hitherto to attempt quantitative assay of estrogens on the basis of this reaction. In the method described below, this difficulty is avoided. By the use of phosphoric rather than sulfuric acid in the reaction solution, the interference of nonspecific response is largely eliminated.¹⁶ Thus a quantitative test possessing a desired degree of specificity is afforded.

Reagents. 1. Phosphoric acid, s. g. 1.75, purest. 2. Crystalline estrogens.[†] 3. Ethanol, pure, redistilled from an all-glass still.

Special Apparatus. 1. Test tubes provided with ground-glass stoppers (capacity 15 cc, Pyrex). 2. Fluorimeter provided with a micro-cuvette (capacity 2 cc).

Procedure. A volume not exceeding 2 cc of the pure alcoholic solution of estrogen

is evaporated from a test-tube over a water bath and oven-dried for one hour at 110°C. To the dry residue, 3.0 cc phosphoric acid is added from a burette. The test-tube, closed with ground-glass stopper, is transferred to a boiling water bath (98°) and heated in the dark for 30 minutes, the mixture being shaken during the first 2 min. of heating to insure the solution of the estrogen. Reaction is interrupted by cooling in tap water. The fluorescence is measured within an hour. The concentration of the estrogen solution is read against a concurrently prepared standard reference curve.

Prolonged exposure of estrogen in acid to light is avoided as light interferes with the development of the fluorescence and also causes gradual fading of developed fluorescence. In performing the reaction, care is taken to prevent accidental entry of water (spray or vapour) into the reaction mixture, as water sharply depresses the reaction and exerts a marked quenching effect. The reaction vessels should be sealed with close-fitting ground-glass stoppers, rubber and cork being unsatisfactory.

Experimental. Pure crystalline estrogens were used for the experiments. The fluorescence was measured in a 2 cc micro-cuvette with a Lumetron photofluorimeter[‡] using a Uviarc mercury vapor lamp equipped with primary filter as for riboflavin assay, and secondary orange filter which transmits freely above 5500 Å. The instrument was calibrated against fluorescein solution of known concentration.

Effect of heating time. The effect of heating time on the intensity of the fluorescent response of estrone and estradiol was determined for the time interval 5-30 minutes. The results in Table I demonstrate that the reaction proceeds regularly with time, rising rapidly in the first 10 minutes, and then less rapidly to a maximum at 20 minutes and is maintained at 30 minutes. Prolonged heating (60 minutes) led to loss of fluorescence. A heating time of 30 minutes seems therefore to be suitable and is employed in all following experiments.

¹¹ Wieland, H., Straub, W., and Dorfmueller, T., *Z. physiol. Chem.*, 1929, **186**, 97.

¹² Marian, G., *Biochem. J.*, 1930, **24**, 1021.

¹³ Schwenk, E., and Hildebrandt, F., *Biochem. Z.*, 1933, **259**, 1021.

¹⁴ Cuboni, E., *Klin. Wchnschr.*, 1934, **8**, 302.

¹⁵ Sala, S. L., cited in *Sex and Internal Secretion*, p. 892, Williams & Wilkins Co., 1939.

¹⁶ Hestrin, S., and Mager, J., *Nature*, 1946, **158**, 95.

[†] Estrone and estradiol were kindly supplied by Dr. B. J. Brent, Roche Organon, Inc.; estriol was obtained through the courtesy of Dr. O. Kamm, Parke Davis Co.

[‡] Thanks are due to Prof. I. S. Friedenwald for his kind gift of a Lumetron apparatus.

Hirschfelder³ reported that after 20-40 g of magnesium sulfate were administered to a human subject, no increase occurred in the magnesium content of the blood, but 40% was excreted in the urine in 24 hours. How much of the sulfate was absorbed was not stated.

Verzar and McDougal⁴ pointed out that the rate of absorption of the sulfate ion is about the same as that of substances of equal weight and compares favorably with that of pentoses and certain hexoses. The data we are presenting show a parallelism between the rates of absorption of the magnesium and the sulfate ions. So far as we are aware, this has not been previously emphasized.

³ Hirschfelder, A. D., *J. Biol. Chem.*, 1934, **101**, 647.

⁴ Verzar, F., and McDougal, E. J., *Absorption from the Intestine*, Longmans, Green & Co., London, 1936.

Magnesium sulfate is widely employed by both practitioner and laymen and is probably one of the most commonly used household cathartics. A number of cases of magnesium poisoning^{5,6} have rather recently been reported, some of which have proved fatal.

Summary. The rate of absorption of magnesium sulfate was studied in a series of barbitalized dogs each of which had suffered a hemorrhage equal to 3% of its body weight. The control animals absorbed 14% of the magnesium and 13% of the sulfate, whereas the experimental animals absorbed 17% and 16% respectively. The differences were not statistically significant. A fact not sufficiently appreciated is that the intestine is only relatively impermeable to this salt.

⁵ Bryon, F. E., *J. Malaya Br., Brit. M. A.*, 1939, **3**, 100.

⁶ Gens, J. P., *J. A. M. A.*, 1943, **123**, 102S.

15701

Estimation of Steroid Estrogens by Fluorimetry.*

M. FINKELSTEIN, S. HESTRIN, AND W. KOCH. (Introduced by Bernhard Zondek.)

From the Hormone Research Laboratory and the Department of Hygiene and Bacteriology, The Hebrew University, Jerusalem.

Estrogens are still generally determined by biological methods. As these present all the difficulties and uncertainties of the biological technic, considerable interest attaches to the development of an adequate chemical assay for the estrogens. During more than a decade repeated attempts have been made to elaborate a colorimetric procedure suitable to this purpose.¹⁻¹⁰ Most of this work has been based on the Kober reaction. The

available colorimetric methods are, however, difficult in practice, of imperfect specificity, and often insufficient in sensitivity. A search in the field of fluorimetry for an adequate solution of this analytical problem would seem to be indicated. The proven high sensitivity of fluorimetric methods in vitamin assay encourages the hope that methods of this type could find usefulness in determination of hormones at biological level.

* This paper was partly aided by a grant from the Max London Foundation administered by Dr. M. Bloom.

¹ Kober, S., *Biochem. Z.*, 1931, **230**, 209.

² Kober, S., *Biochem. J.*, 1938, **32**, 357.

³ Cartland, F. G., Meyer, R. K., Miller, L. C., and Rutz, M. H., *J. Biol. Chem.*, 1935, **109**, 213.

⁴ Venning, E. H., Evelyn, K. A., Harkness, E. V., and Browne, Y. S. L., *J. Biol. Chem.*, 1937, **120**, 225.

⁵ Bachman, C., *J. Biol. Chem.*, 1939, **131**, 455.

⁶ Bachman, C., and Pettit, D. S., *J. Biol. Chem.*, 1941, **138**, 689.

⁷ Talbot, N. B., Wolfe, Y. K., MacLachlan, E. A., Karush, F., and Butler, A. M., *J. Biol. Chem.*, 1940, **134**, 319.

⁸ David, K., *Acta brev. Neerland*, 1934, **4**, 64.

⁹ Zimmermann, W., *Z. physiol. Chem.*, 1935, **233**, 257; 1936-37, **245**, 47.

¹⁰ Pineus, G., Wheeler, G., Young, G., and Zahl, P. A., *J. Biol. Chem.*, 1936, **116**, 253.

TABLE II.
Analyses of Estrogen Solutions by the Fluorimetric Method.

Estrogen	Conc. per cc in γ as given	Conc. per cc in γ as read	Error of estimation in γ
Estrone	0.00	0.0	0.0
	6.50	6.5	0.0
	8.50	8.3	-0.2
	9.00	8.9	-0.1
	9.80	8.95	-0.85
	10.00	9.75	-0.25
	10.00	9.80	-0.20
Estradiol	1.50	1.46	-0.04
	2.20	2.15	-0.05
	2.40	2.40	-0.00
	4.30	4.22	-0.08
Estriol	1.50	1.5	0.0
	1.50	1.65	+0.15
	4.00	3.8	-0.2
	4.00	4.0	0.0

Effect of estrogen concentration. Typical concentration response curves are shown in Chart 1. It can be seen that the intensity of fluorescence is a linear function of estrogen concentration to at least 2.5 γ of estradiol, and 5 γ of estrone. The curve for estriol seems to be slightly convex to about 2 γ , then straightens out and follows a linear course from 3 γ to at least 10 γ . The estriol solutions have a faint pink color; the estrone and estradiol solutions are virtually colorless. Weight for weight, estradiol is 3.6 times and estrone about 1.3 times as active in production of the fluorescence as estriol. The sensitivity of the method therefore varies with the estrogen determined.

Results obtained on solutions of pure estrogen in concentrations unknown to the authors at the time of the analyses are shown in Table II. In optimum ranges of estrogen concentration and when each test was performed in duplicate on 2 dilutions of the test solution, the mean error of estimation in a series of 30 determinations proved to be $\pm 3\%$.

Preparation of standard curve. The instrument was calibrated against sodium fluoresceinate so that a solution of a concentration of 0.8 γ /cc rated near 40 on the galvanometer scale and precisely 100 on the potentiometric scale.

Calibrations were made both before each series of measurements and during measure-

ments at intervals of 10 minutes. Standard curves were prepared from parallel measurements on graded estrogen concentrations so selected to fall within the potentiometric range 10-90. The ordinary scatter of results in a parallel replicate series can be seen in Fig. 1. Standard curves obtained on different days using the same batch of phosphoric acid fluctuated within $\pm 5\%$. A daily check of the standard curve is recommended for accurate work.

Specificity. Effective use of the fluorescence reaction as the basis of estrogen assay in biological materials is dependent on proof of the reaction's specificity as between different ether-soluble biological substances which tend to accompany the estrogens in usual procedures for their extraction.

Nonestrogenic mononuclear urinary phenols (tyrosine, phenol, and cresol) showed in an amount of 1 mg neither fluorescence nor color reaction under the conditions of the test.

The behaviour of 13 sterols[§] listed in order of decreasing activity is recorded in Table III. It may be seen that of the examined nonestrogenic sterols only desoxycorticosterone and testosterone have sufficient activity to produce a false positive estrogen reading under the conditions of the test. As

[§] The authors are indebted to Dr. F. Bergman, Daniel Sieff Research Institute, Rehovot, for a kind gift of sterols.

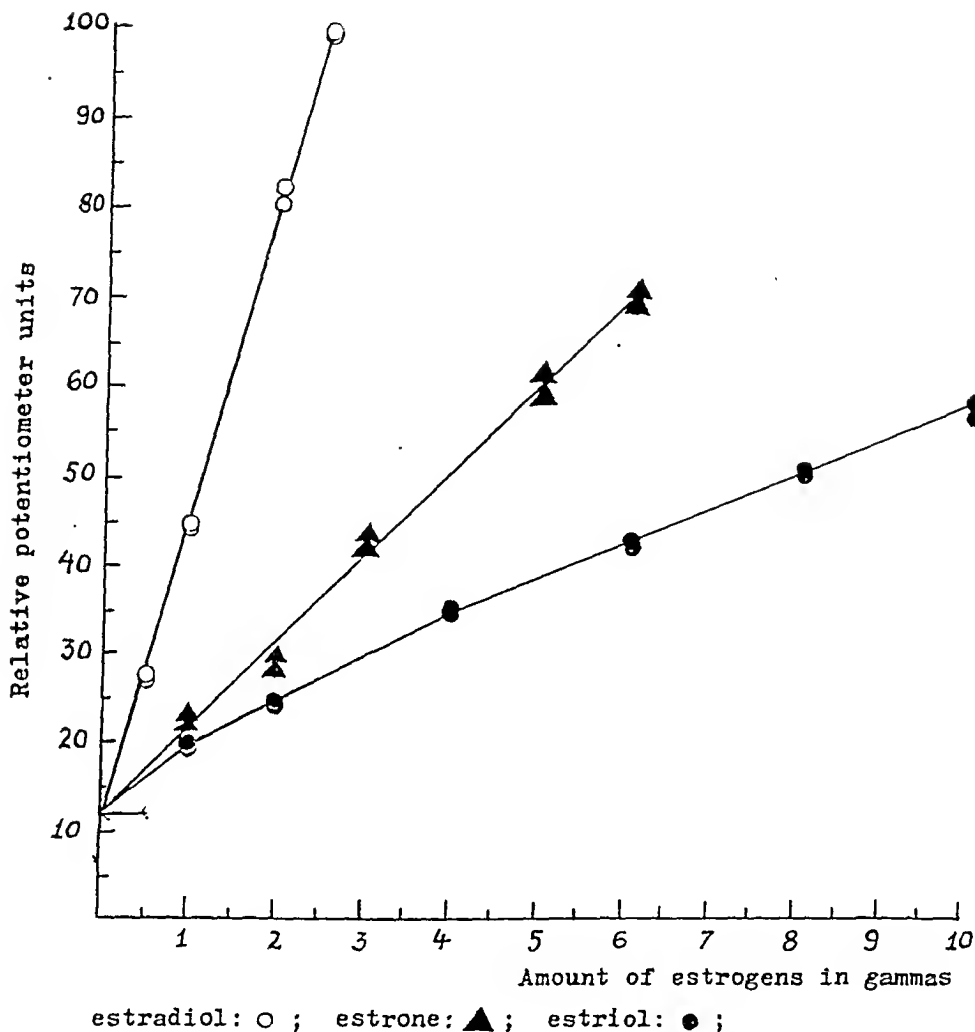


Chart 1.

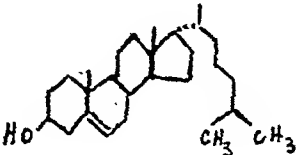
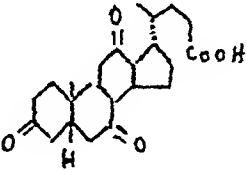
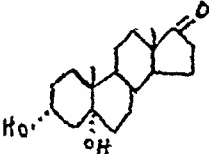
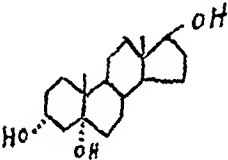
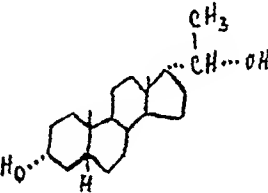
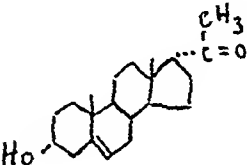
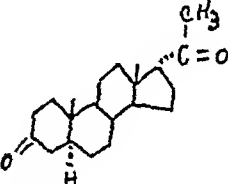
Influence of estrogen. concentration on fluorescence response.

The ordinate intercept represents an intrinsic instrument factor.

TABLE I.
Effect of Heating Time in Phosphoric Acid on Fluorescence Reaction of Estrone and Estradiol.

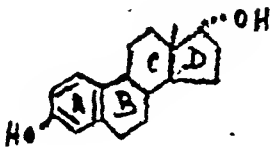
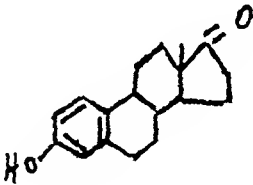
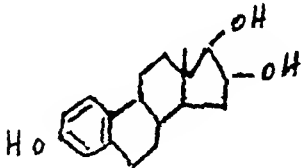
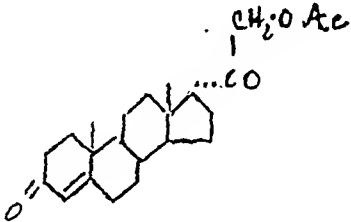
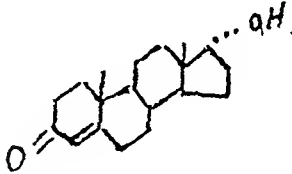
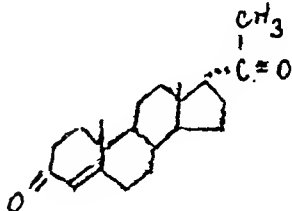
Substances	Fluorescence measurement in relative galvanometric units at different times			
	5'	10'	20'	30'
Estrone. 3 γ	27.5	35.5	56.0	57.5
Estradiol, 1 γ	17.0	31.0	40.0	42.5

TABLE III. (Continued).

No.	Substance	Structural formula	Fluorescence activity per γ relative to estrone = 1
7	Cholesterol		0
8	Dehydrocholic acid		0
9	Androsterone		0
10	Androstenediol		0
11	Pregnanediol		0
12	Pregnenolone		0
13	Allo-pregnanedione		0

* The structural formulae conform to the conventions adopted by H. Selye (*Encyclopedia of Endocrinology*, Franks Publishing Co., Montreal, 1943).

TABLE III.
Correlation of Chemical Structure and Fluorescence Response to Heating in Phosphoric Acid.*

No.	Substance	Structural formula	Fluorescence activity per γ relative to estrone = 1.
1	Estradiol		2.7
2	Estrone		1.0
3	Estriol		0.75
4	Desoxycorticosterone-acetate		0.3
5	Testosterone		0.1
6	Progesterone		0.01

action to estrogen assay in a material which contains several estrogens must entail prior separation of the different components. Several recommended fractionation technics fortunately meet this requirement.^{15,18-20} It may be noted here that the nonsteroid estrogen, stilbestrol, does not produce fluorescence under the conditions of the test.

The fluorimetric method is simple, the reproducibility of the result is good, and the specificity of the test promises to be as good as or better than has been obtained with the previously described colorimetric methods. Further study of the analytical possibilities of this assay seems therefore to be desirable. A survey of the effect of phosphoric acid on a larger number of sterols and related substances with a view to elucidating the relation of sterol structure to fluorescent response has accordingly been undertaken. At the same time the application of the fluorimetric method to estrogen determination in blood

and urine is being examined. The results will be reported elsewhere.

Summary. 1. A method for estimation of estrone, estradiol, and estriol in pure solution is based on the observation that these substances afford products with a green fluorescence when heated in phosphoric acid.

2. The fluorescent response of the urinary estrogens is over a wide range a linear function of their concentration. The error of the fluorimetric assay in suitable ranges of estrogen concentrations (estradiol, 0.5-2.5 γ ; estrone, 1.0-5.0 γ ; and estriol, 1.0-10.0 γ) does not exceed $\pm 3\%$.

3. Sterols which do not contain a conjugated double bond, some naturally occurring nonsteroid aromatic substances, and many common constituents of biological material give no fluorescent response under the conditions of the test.

4. Estrogen fluorimetry appears to compare favorably as to sensitivity, specificity and simplicity with previously described methods of estrogen assay.

The authors wish to express their indebtedness to Prof. B. Zondek, for his kind interest and support of this investigation.

¹⁵ Mather, A., *J. Biol. Chem.*, 1942, 144, 617.

¹⁹ Pineus, G., and Pearlman, W. H., *Endocrinology*, 1942, 31, 507.

²⁰ Scimmel, B. F., *J. Biol. Chem.*, 1946, 162, 99.

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Induction of Renal Glomerular Lesions by Urethane in Inbred Mice Susceptible to Spontaneous Glomerulonephritis.*

ARTHUR KIRSCHBAUM AND E. T. BELL.

From the Departments of Anatomy and Pathology, University of Minnesota Medical School, Minneapolis, Minn.

Mice of the NH strain develop spontaneous glomerulonephritis. Histologically the glomeruli are similar to those of the human chronic disease.¹

* This investigation has been aided by a grant from the Life Insurance Medical Research Fund. The authors are indebted to Miss Maribella Frantz, Miss Elvora Stahn, and Mrs. Sonia Braun for valuable technical assistance.

¹ Kirschbaum, A., *Proc. Soc. Exp. Biol. and Med.*, 1944, 53, 250.

² Dunn, T. B., and Larsen, C. D., *Fed. Proc.*, Part I, 1946.

Dunn and Larsen² reported that urethane induced hyaline alterations in the glomeruli of mice of the Strong A strain, whereas similar changes were lacking in the glomeruli of other stocks of mice following the administration of this drug.

The work of Dunn and Larsen suggested to the authors that urethane might provoke interesting changes in the glomeruli of mice which are susceptible to the development of spontaneous glomerulonephritis. Twenty-one NH mice received 1 mg per g of body weight of urethane (anesthetic dose) in

neither of these 2 possible interferants is a phenol, their separation from the urinary estrogens presents no great difficulty.

In test-tube experiments on a random collection of substances, the following results were recorded. Tryptophane and quinine showed marked fluorescence. Stilbestrol, catechol, all amino acids other than tryptophane, glucose and polyglucosides, several purines and polyhydric alcohols showed no reaction. Carbohydrates containing a non-glucose sugar constituent gave amber reactions but no fluorescence.¹⁷

Discussion. The fluorescence reaction described seems suitable for use as the basis of an estrogen assay. Furthermore this reaction may afford also a basis of assay for certain nonestrogenic sterols, *e.g.* testosterone and desoxycorticosterone acetate. The fluorescence response of these latter, though smaller than that of the estrogens, is readily measurable at a level of about 10 γ . On the other hand, the minimum amounts of estrogen that can be determined in terms of fluorescence reaction within an error range of $\pm 3\%$ are for estrone 1 γ or 10 I.U., for estradiol 0.5 γ or about 5 I.U., and for estriol 1 γ or about 0.01 I.U. Thus the sensitivity of the fluorescence assay in the case of estriol exceeds that of the biological test and in all the cases exceeds that of known colorimetric procedures.¹⁻¹⁰

When estrogens are heated with sulfuric acid, a fluorescence exceeding in intensity that afforded by phosphoric acid is obtained. In principle, therefore, sulfuric acid might seem preferable to phosphoric acid as the reaction agent. When sulfuric acid is used, however, blank fluorescence of the reagent complicates the reading, color changes obscure the determination, the results are less reproducible, and greater technical inconvenience is encountered. For quantitative work, phosphoric acid seems decidedly to be preferable.

The experiments reported show that fluorescent response to heating in phosphoric acid is a specific characteristic of certain sterols. A consideration of the structural

formulae of the investigated sterols (Table III) leads to certain generalizations concerning structural features which mediate the production of fluorescence under the conditions of this test. It is evident from the data that the investigated sterols fall into 2 groups:

(a) Substances Nos. 1-6 which show a definite fluorescent response.

(b) Substances Nos. 7-13 which show no measurable fluorescent response.

All the substances of Group (a) possess a conjugated double-bond system and have this system in ring A of their structure. Two types of conjugated double-bond systems are exemplified in this group: aromatic or polyenoid (C:C:C:C), *e.g.* substances Nos. 1-3; and catienoid (C:C:C:O), *e.g.* substances Nos. 4-6. On the other hand, no member of Group (b) possesses a conjugated double bond, although some of the members of this group do possess one or more nonconjugated double bonds. It is suggested therefore that the presence in the sterol molecule of a conjugated double bond is a prerequisite of the fluorescent activity. From the fact that the magnitude of the response of different substances in Group (a) varies markedly, it must be concluded that the magnitude of a fluorescent response is determined not merely by the presence of a conjugated double bond but also by other characteristics of the molecule. It is evident that the presence and position of polar groups exerts an important influence on the magnitude of the fluorescent response. High activity such as the urinary estrogens exhibit appears to be associated with aromatic phenol character in ring A and presence and position of oxygen bonds in ring D (Table III).

As may be seen below, the biological activity and fluorescent response of the investigated estrogen trio are correlated, though by no means perfectly.

Relative activity	Estriol	Estrone	Estradiol
Estrogenic	0.01	1	5
Fluorimetric	0.75	1	2.7

Since the ratio of estrogenic activity to fluorimetric response per gamma is a variable, it is clear that the application of the re-

¹⁷ Hestrin, S., and Mager, J., 1946, unpublished.

Coproporphyrinuria (Type III) in Acute Poliomyelitis.*

C. J. WATSON, WM. SCHULZE, VIOLET HAWKINSON, AND A. B. BAKER.

From the Departments of Medicine and Neuropsychiatry, University of Minnesota Hospitals, Minneapolis, Minnesota.

The recent epidemic of poliomyelitis in Minneapolis has provided opportunity to investigate the urinary coproporphyrin excretion in this disease. The need for such a study was suggested by a number of facts relating to the normal and pathological physiology of the porphyrins. Chief among these was the observation of Klüber,^{1,2} that the white matter of the central nervous system of warm-blooded animals regularly contains a small amount of coproporphyrin. Klüber's study[†] indicated that this was probably the Type III isomer, at least from the standpoint of its solubility characteristics. Our own studies bearing on this point, to be described in detail elsewhere, have likewise indicated the presence of the Type III isomer, on the basis of the differential precipitation or "fluorescence quenching" method.³

In addition to the physiological occurrence of coproporphyrin in the central nervous system, the markedly increased urinary excretion of the Type III isomer in lead and arsenic poisoning, and, in association with uroporphyrin, in intermittent acute porphyria (diseases in which the nervous system is commonly affected), constituted additional reasons for investigating the porphyrin metabolism in poliomyelitis. This disease has in fact been confused clinically with acute porphyria,⁴ an additional instance of the latter type having come to our attention in recent weeks.

Still another reason for investigating the urinary coproporphyrin in poliomyelitis is the occurrence of unexplained hypertension in this disease, especially in the bulbar type. This has been observed rather frequently in the present epidemic. So far as can be determined, hypertension in poliomyelitis has been mentioned specifically in but one report in literature (Salus⁵) although it has been referred to in association with lesions of the brain stem.⁶ It is well known, of course, that hypertension is a relatively common manifestation of intermittent acute porphyria, with or without bulbar manifestations, although the relationship is as yet unexplained. Carrié⁷ classifies the hypertension of porphyria, and of lead poisoning, as vasospastic in type, designating it as "porphyrinopathic."

The present material consisted of 64 cases of poliomyelitis studied at various stages of the disease, and representing various types and degrees of severity. One hundred and ninety determinations of total coproporphyrin were made in these cases, on aliquots of 24-hour urine samples, a modification of Fikentscher's method⁸ being used.[‡] Fifty-one isomer analyses[‡] were made by means of the differential precipitation of the esters of coproporphyrin I and III in 30-35% acetone in the cold.^{3,9} The results obtained

⁵ Salus, F., *Klin. Wchnschr.*, 1932, **11**, 1542.

⁶ Page, I. H., and Coreoran, A. C., *Arterial Hypertension. Its Diagnosis and Treatment*, Year Book Publishers, Chicago, 1945, p. 20.

⁷ Carrié, C., *Die Porphyrine, Ihr Nachweis, Ihre Physiologie und Klinik*, Leipzig, G. Thieme, 1936.

⁸ Fikentscher, R., *Biochem. Z.*, 1932, **249**, 257.

[‡] Details of these procedures will be given in a forthcoming publication.⁹ Of the two alternative methods of isomer analysis, A and B, the latter was used in the present study.

⁹ Schwartz, S., Hawkinson, V., Cohen, S., and Watson, C. J., *J. Biol. Chem.*, in press.

* Aided by a grant from the John and Mary R. Markle Foundation, New York City, and the National Foundation for Infantile Paralysis, Inc.

¹ Klüber, H., *J. Psychol.*, 1944, **17**, 209.

² Klüber, H., *Science*, 1944, **99**, 482.

[†] Personal communication.

³ Schwartz, S., Hawkinson, V., and Watson, C. J., *Science*, 1946, **103**, 338.

⁴ Waldenström, J., *Acta Psychiatrica et Neurologica*, 1939, **14**, 375.

aqueous solution once each week by subcutaneous injection. The mice were 6 to 8 weeks of age when the experiment was begun.

Between 5 and 6 months after the series of injections was started 11 of the mice became grossly edematous. The plasma protein levels of these animals were depressed (values of less than 5.2 g%,³ the lowest level obtained for the mice without edema), the urine contained albumin, and blood urea nitrogen was elevated (range of from 50 to 265 mg% as compared with a normal of 18-27 mg%).⁴ In one case there was a gross hyperlipemia; the plasma cholesterol value was 430 mg% as compared with a normal for mice of approximately 100 mg%.⁵ All the injected mice were anemic. The degree of nitrogen retention, but not of anemia, could be correlated with the extent of the pathologic alterations observed in the glomeruli.

The essential pathologic changes in the glomeruli were thickening of the capillary basement membrane (azo-carmin stain), capillary thrombosis, and closure of many capillary tufts resulting from the basement membrane alteration. The affected glomeruli were smaller than those observed in spontaneous glomerulonephritis of NH mice, more glomeruli were normal, and except in an occasional glomerulus there was nothing to suggest endothelial proliferation. Histologically the glomeruli resembled those of human lipoid nephrosis.⁶ In one case most of the glomeruli were extensively infiltrated with polymorphonuclear leukocytes.

Of the 21 mice injected with urethane, either edema or histologic evidence of renal

disease and nitrogen retention were observed in 17. One animal was not nephritic as judged by gross, histologic and biochemical evidence; 3 were without renal disease grossly and the kidneys were not studied histologically because of postmortem degeneration.

Spontaneous glomerulonephritis appears rarely before the age of 8 months in NH mice. Of 53 cases in a total of 199 controls only 5 appeared before 8 months of age. There is no doubt that the glomerular lesions were induced by urethane; the effect of urethane on the kidney was confined to the glomerulus.

Urethane is a capillary poison.⁷⁻⁹ In NH mice the glomerular capillaries especially seem to be susceptible to its effects. The drug also induces lung tumor development in mice,¹⁰ and most of the animals of this experiment developed multiple lung adenomas. It is likely that the anemia was not entirely the result of the renal disease. Urethane depresses the myeloid tissues of mice,¹¹ and, furthermore, the injected animals failed to gain in weight to the same extent as normal mice. These animals might belong in the same category as underfed mice, which are characteristically anemic.

Summary. Urethane induced glomerular lesions in NH mice, an inbred strain which is susceptible to the spontaneous development of glomerulonephritis. Mice with the urethane-induced glomerular alterations exhibited renal insufficiency, albuminuria, edema, and depressed plasma protein levels. One case evidenced hypercholesterolemia.

³ Koch, F. C., and McMeekin, T. L., *J. Am. Chem. Soc.*, 1924, **46**, 2066 (method for determining plasma proteins).

⁴ Karr, W. G., *J. Lab. Clin. Med.*, 1924, **9**, 329 (method for determining blood urea nitrogen).

⁵ Bloor, W. R., *J. Biol. Chem.*, 1928, **77**, 53 (method for determining plasma cholesterol).

⁶ Bell, E. T., *Am. J. Path.*, 1938, **14**, 691.

⁷ Krogh, A., and Harrop, G. A., *J. Physiol.*, 1920, **54**, CXXV.

⁸ Landis, E. M., *Am. J. Physiol.*, 1927, **81**, 124.

⁹ Doljanski, L., and Rosin, A., *Am. J. Path.*, 1944, **20**, 945.

¹⁰ Nettleship, A., and Henshaw, P. S., *J. Nat. Cancer Inst.*, 1943, **4**, 309.

¹¹ Engstrom, R., Kirschbaum, A., and Mixer, H. W., *Science*, in press.

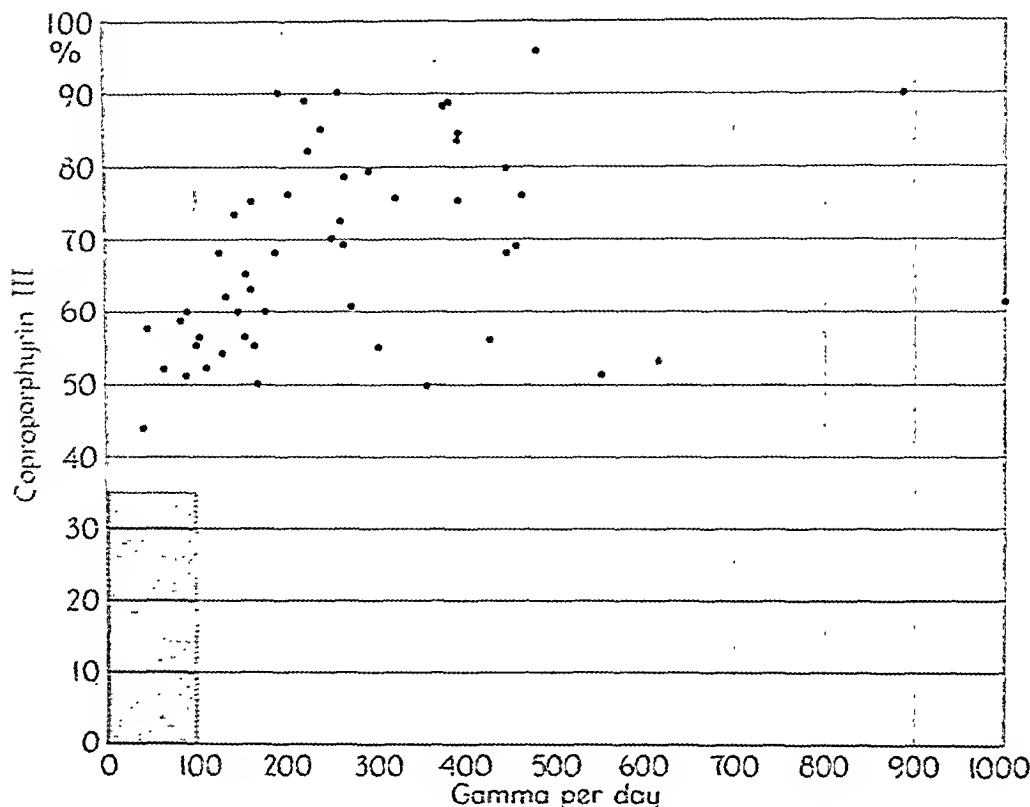


FIG. 2.

Total coproporphyrin (abscissa) and percentage of type III isomer (ordinate) in 38 cases (51 analyses). The shaded area in the lower left corner represents the normal range.

also by direct isolation¹¹ from five 24-hour urine samples, 2 from Case 7, and 1 each from Cases 1, 2 and 59 (case numbers refer to Fig. 1).

The results of these isolations are given in Table I, in which it is evident that the Type III coproporphyrin isomer is clearly present in excess.

One case, which was not included among the above, deserves special comment. In this instance (L.H., ♂, 32), despite a value of 6.2 γ for total urinary coproporphyrin, the isomer analysis revealed but 18% of Type III. This patient differed from all the others, however, in having a mild jaundice, and in having been exposed to 2 cases of jaundice among the members of her own family, about one month previously. In addition to jaundice, she exhibited a tender liver for a short

period; also, evidence of hepatic functional impairment including urobilinogenuria, positive cephalin-cholesterol flocculation and thymol turbidity tests, and abnormal bromsulphalein retention. While all the latter might possibly be regarded as due to toxic injury of the liver secondary to poliomyelitis, the presence of jaundice, the history of close contact with infectious jaundice, and the tender liver, strongly favored a specific virus hepatitis. Yet there was also evidence suggestive of poliomyelitis. The neck flexion sign was strongly positive, there was muscle weakness, especially in the deltoids and triceps, and the spinal fluid contained 17 cells per cu mm (100% lymphocytes). Although, as noted above, the majority of the increased coproporphyrin was Type I, the actual amount of coproporphyrin III in the 24-hour sample, i.e., 1.17 γ , is far above the normal. Thus, it is evident that both isomers

¹¹ Grinstein, M., Schwartz, S., and Watson, C. J., *J. Biol. Chem.*, 1945, 157, 323.

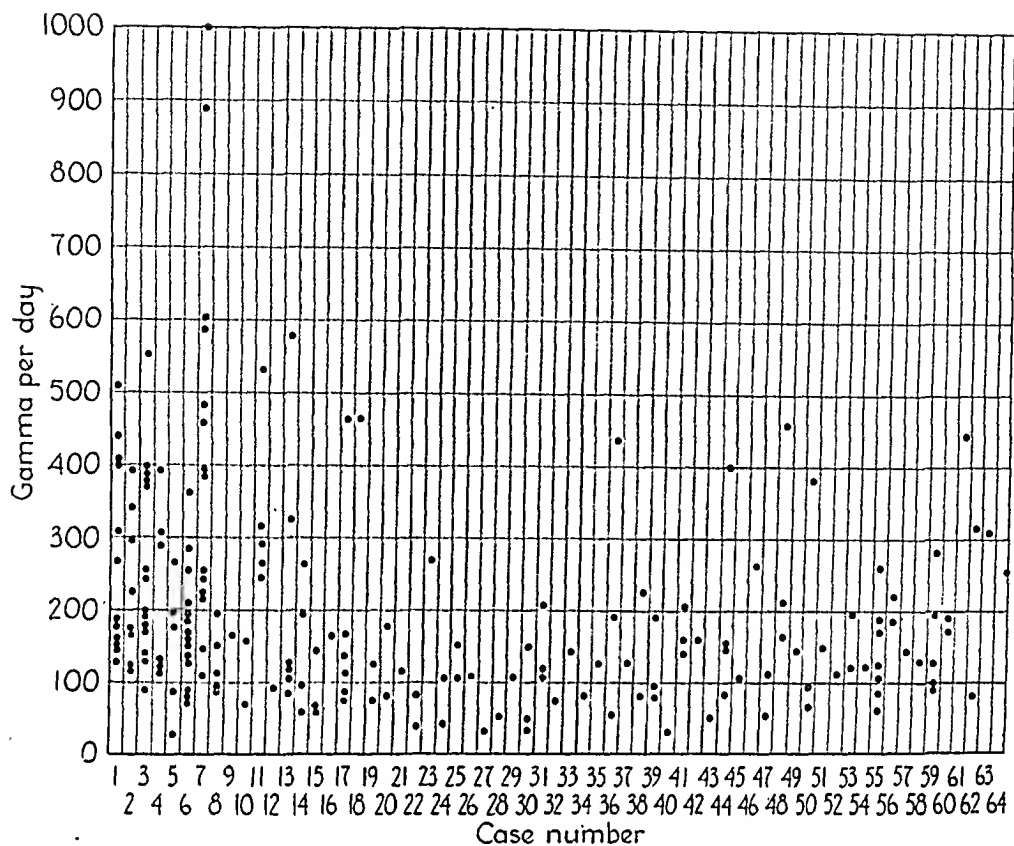


Fig. 1.

Values for total coproporphyrin in γ per 24 hours, as determined 190 times in 64 cases.

have been sufficiently uniform that there can be little doubt about the essential phenomenon which they represent, whatever its significance. The purpose of the present report is to record these observations.

In Fig. 1, the results of the total coproporphyrin determinations are shown as carried out on the 24-hour urine samples. In Fig. 2, 51 isomer analyses as carried out on 38 cases, have been plotted in terms of both total coproporphyrin and percentage of the Type III isomer, as compared with the normal range (the shaded area in the left lower corner of Fig. 2).

Data have been presented,¹⁰ and these will be considered in more detail elsewhere, which reveal that the normal range of urinary co-

porphyrin in adult human beings is from 20-100 γ per day, of which 8 to 35% is the Type III isomer, the remainder being Type I. Comparison of these figures with those given above in Fig. 1 and 2 reveal the frequency of increase of the total coproporphyrin and of the proportion of the Type III isomer which characterize the urine in cases of poliomyelitis.

Since the possibility existed that some peculiarity of the urine in these cases might be affecting the differential precipitation technic in such a way that Type I isomer was behaving in part like Type III, it was clearly necessary to control the observations by means of isolation of the crystalline coproporphyrin ester and determination of its melting point. This has been done from the pooled final solutions used for isomer analysis, as obtained from 19 urine samples;

¹⁰ Watson, C. J., Proc. Am. Soc. Clin. Invest., 1946, *J. Clin. Invest.*, in press.

In Case 7, in which the highest levels were noted, the first determination, on the 9th day of the disease, was 216 γ , after which it increased to 392 γ on the 11th, 455 γ on the 13th, 890 γ on the 14th, then fell to an almost normal level of 105 on the 20th day only to rise again to a 1000 γ level on the 23rd day, without any marked change in the clinical condition of the patient. The level then again fell to 146 γ on the 26th day of the disease with exitus on the 30th day after onset.

In Case 3, determinations were made from the 20th to the 44th day of the disease, with levels ranging from 195 γ on the 20th day to a high of 550 γ on the 26th day, with several subsequent rises and falls and finally a decrease to the normal range on the 44th day after onset. This patient went on to a good clinical recovery.

It is thus apparent that the urinary coproporphyrin values have not been of assistance in prognosis nor could we correlate them with the current clinical status of the patient. Insofar as hypertension was concerned, there was also little, if any, correlation. It was true that in nearly all cases with elevated blood pressure in which porphyrin determinations were made, increased amounts were found. The only 2 exceptions were in pregnant women whose acute attacks were 3-4 months in the background and whose hypertension was believed to be on the basis of toxemia of pregnancy. There were many individuals, however, whose blood pressure was normal in spite of markedly increased porphyrin values. If the elevated blood pressure is "porphyriopathic" it would have to be assumed that constitutional or other factors were responsible for the differences encountered. In one instance the blood pressure and porphyrin values were noted to decrease concomitantly, but in other cases marked fluctuations in blood pressure were recorded without evident correlation with the urinary porphyrin values.

Fever^{12,13} and epidemic hepatitis,¹⁰⁻¹³ as well as ordinary hepatic functional dis-

turbances, whether parenchymal or mechanical in type,¹⁰⁻¹³ have been shown to be associated with an increase of the Type I coproporphyrin isomer in the urine. Until now, infectious disease has not been found to cause an increased excretion of coproporphyrin III. It is of much interest in this connection that the increase in cases of infectious hepatitis is regularly due to the Type I isomer,¹⁰ yet the virus of this disease is similar in some respects to the virus of poliomyelitis, both being relatively resistant and both being found in the gastrointestinal tract. Thus, it would appear logical to seek for an explanation of the difference in porphyrin excretion in the 2 diseases in the effect on the organism of the neurotropic as versus the hepatotropic nature of the respective viruses. The possibility must be considered that the excessive coproporphyrin III excretion observed in the present cases of poliomyelitis is in some way related to a disturbance of the coproporphyrin of the central nervous system. Another possibility is that it is derived from myohemoglobin, as a result of the disturbance in muscle physiology. This appears unlikely in view of the fact that marked increases have been noted in cases of relatively pure bulbar type with little or no muscle paralysis, also in early cases prior to any demonstrable muscle weakness.

Inspection of the isomer data shown in Fig. 2 reveals that while coproporphyrin III is clearly preponderant there is also, in many instances, a distinct increase of the Type I isomer. The simultaneous occurrence of marked urobilinogenuria in a number of these cases suggests that the increase in coproporphyrin I may be due to hepatic functional impairment.

Further studies are in progress with respect to the significance of these observations. The increased excretion of coproporphyrin III establishes a biochemical difference between 2 specific virus diseases, *i.e.*, poliomyelitis and infectious hepatitis. It is desirable to ascertain whether other neurotropic virus affections are similarly characterized, and whether virus diseases of dissimilar type, such as atypical pneumonia or psittacosis, if they exhibit any increases in

¹² Watson, C. J., *J. Clin. Invest.*, 1936, **15**, 327.

¹³ Dobriner, K., *J. Biol. Chem.*, 1936, **113**, 1.

TABLE I.
Percentage of Type III Isomer, and Melting Points of Crystalline Methyl Esters Isolated from Various Cases in the Series.

Case No.	Total coproporphyrin in γ per 24 hr	% of Type III isomer	Crystal habitus	Melting point ($^{\circ}$ C) of crystalline methyl ester		Melting point ($^{\circ}$ C) of Cu complex of methyl ester	
				1st crop	1st recryst.	1st crop	Recryst.
1. (H.M.)	441	80	Rosettes of prisms*	130-151	128-135		
2. (J.D.)	390	75	" "	130-142	130-132	200-210	201-209
5b. (W.S.)	378	88	" "	131-143	130-131		
7. (K.P.)	1000	62	" "	160-183	152-160	199-209	200-206
7. (K.P.)	890	90	" "	130-141	130-134		
45-63†			" "	150-183	140-142		
			" "		130-132		

* The tetramethyl ester of coproporphyrin III crystallizes characteristically in rosettes of straight prisms which exhibit dimorphism in melting point: 135 $^{\circ}$; 144 $^{\circ}$ C; 167-170 $^{\circ}$ C.††

† Pooled final solutions used for total coproporphyrin determinations.

‡ Pooled crystals from cases 1, 2, and 5b, were converted to the Cu complex†† which crystallized from CHCl_3 : CH_3OH after preliminary chromatographic purification.†† The Cu complex of coproporphyrin III methyl ester melts at 206-207 $^{\circ}$ C (corrected). All of the above melting points are corrected.

were increased, a finding which is fully compatible with the belief that the patient had both diseases simultaneously.

Discussion. The data thus far obtained do not indicate the existence, in poliomyelitis, of a correlation of the degree of increase of the urinary coproporphyrin with severity of the disease, prognosis as to death or recovery, or extent of residual paralysis; or as to type of disease, whether bulbar, spinal respiratory, or mixed. Nevertheless, in the 10 fatal cases which have been studied, all the determinations of the total coproporphyrin and the percentage of Type III isomer were significantly, and as a rule markedly, elevated regardless of the stage of the disease. In a number of instances, high values have been encountered for many days and in some cases for a number of weeks beyond the febrile period, likewise in some cases who have had a relatively mild attack and are now recovering. The majority of the values below 100 γ per 24 hours, as noted in Fig. 1, were obtained during the convalescent period. With reference to Fig. 1, it may also be noted that the levels of coproporphyrin excretion in an individual patient have often shown wide variation.

For example, in Case 1, the first determination made on the 4th day of the disease was 186 γ after which it fell to 125 γ and 154 γ on the 6th and 8th days after onset, then rose to 306 γ on the 9th day only to fall again to approximately 150 γ for 3 days, with a further rise to 400 γ from the 13th to 16th days after onset. On the 19th day the value was 264 γ and this was followed by a rise to the highest level of 505 γ on the 21st day. The last value obtained was 441 γ on the 24th day, death ensuing on the 27th day.

By contrast, in Case 2, the highest levels were obtained on the 3rd through the 9th days of the disease with a progressive fall through the 16th day of the disease to a level of 120 γ , death ensuing on the 19th day after onset.

TABLE I.
Analysis of Fetal and Maternal Fluids.

Tests	Fetal fluids		Maternal fluids	
	No. of observations	Quantities and ratios	No. of observations	Quantities and ratios
Amt of urine in individual bladders (cc)	50	0.05-0.1* (1.013-1.018)†	30	(1.0-1.5) (1.026-1.030)
Specific gravity of urine	9	1.015‡ (11.7-23.0)	4	1.029 (12.1-19.0)
Blood urea N (mg/100 cc)	9	16.73 (61-384)	10	15.9 (454-556)
Urine urea N (mg/100 cc)	8	195.0 (3.6-23.0)	3	497.0 (29.0-35.0)
Ratio urine urea/blood urea	8	11.7 (0.7-1.2)	3	31.0
Blood creatinine (mg/100 cc)	3	1.0		
Urine creatinine (mg/100 cc)	1	42.0		
Ratio urine creatinine/blood creatinine	1	42.0		
Urine chlorides, as NaCl (mg/100 cc)	2	178.0	2	265.0
Apparent glucose in urine (mg/100 cc)	5	40-50	7	50-60

* In the males. (In females the bladder was usually empty since the normal hypospadias militates against blocking the urinary outlet by ligating the urinary papilla.)

† Range.

‡ Average.

methods: blood urea—incubation of blood with equal amount of urease suspension and determination of ammonia by nesslerization on Folin-Wu filtrate thereof;⁴ urine urea—determination of ammonia by nesslerization before and after incubation with urease;⁴ creatinine in blood;⁵ creatinine in urine;⁶ chlorides in urine;⁷ specific gravity;⁸ reducing substances in urine—Benedict's qualitative reagent with raw urine, the degree of reduction being gauged against known standards of glucose.

The suitability of these micro-modifications was established by work with pure solutions and by duplicate recoveries from the urine and blood of known small added amounts of the substances in question.

Table I gives the principal data obtained. That the fetal kidneys are functioning as such and not merely producing a transudate

is shown by the ability of the kidney to concentrate certain materials and to reabsorb others.

Since the specific gravity of ultrafiltrates of mammalian plasma is approximately 1.010, the average specific gravity of 1.015 for the fluid from the fetal bladder indicates that overall concentration has occurred. Otherwise stated, this is evidence of active reabsorption of water. If the principle of Long's coefficient is applied, the indication is that there has been in the urine a 50% increase in total solids over that of the glomerular filtrate.

The approximately 12-fold concentration of urea is distinctly less than the 31-fold concentration occurring simultaneously in the mother. However, when it is recalled that the placenta is functioning as an excretory organ, definite evidence is provided that the fetal kidney is already assuming a postnatal role. In this connection, it may be pointed out that the average blood urea nitrogen (b.u.n.) of the fetuses was 0.83 mg% lower than that of their mothers. Had the b.u.n. of the fetus been higher than that of the mother, it might have been taken as evidence of beginning separation of the placenta and such elevation of the fetal b.u.n. might have been considered as a possible

⁴ Peters, J. P., and Van Slyke, D. D., *Quantitative Clinical Chemistry*, Williams and Wilkins, Baltimore, 1932, Vol. 2, vii + 957 pp.

⁵ Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, **38**, 81.

⁶ Folin, O., *J. Biol. Chem.*, 1914, **17**, 469.

⁷ Schales, O., and Schales, S. S., *J. Biol. Chem.*, 1941, **140**, 879.

⁸ Barbour, H. G., and Hamilton, W. F., *J. Biol. Chem.*, 1926, **69**, 625.

urinary porphyrin at all, are, like infectious hepatitis, characterized by an increase of the Type I isomer.

Summary and Conclusion. A considerable excess in the excretion of coproporphyrin III has been commonly noted in a series of urine samples from cases of acute poliomyelitis. The total urinary coproporphyrin

is usually in the range of 100-500 γ per 24-hour sample, with from 50-90% of Type III isomer, as compared with 20-100 γ and 8-35%, normally. This abnormal excretion is contrasted with the marked increase of Type I isomer previously found to characterize the urine in cases of infectious hepatitis.

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Experimental Evidence of the Secretion of Urine by the Fetal Kidney.*

HARRIET DALY, L. J. WELLS, AND GERALD EVANS.
(With the assistance of Dorothy N. Highby.)

From the Department of Anatomy and the Hospital Laboratories, University of Minnesota School of Medicine, Minneapolis.

In the fetal rat, ligation of the ureter causes hydronephrosis and ligation of the urogenital papilla causes filling of the bladder.¹ The rate of formation of fluid by the fetal kidney is surprisingly rapid and it may be accelerated experimentally by ligating the renal pedicles of the mother or by injecting a highly concentrated solution of urea under the skin of the fetus.²

The present study which grew out of the experiments noted above, has been designed to determine whether this fluid is actually urine or merely a transudate of the fetal blood. In order to attain this objective, we have studied the blood and contents of the bladder of the fetus and also those of the mother.

The material for analysis was collected in the Department of Anatomy from rats in which the pregnancies were dated from the moment of observation of coitus. On the 21st day of pregnancy (20 days and 17 hours \pm 2 hours), the mother was anesthetized with ether and her abdomen was opened surgically. Each fetus of a litter was transferred to the abdominal cavity of the

mother³ and its urogenital papilla was ligated by means of an unraveled strand of surgical silk. Then the abdominal incision of the mother was closed by suturing and the anesthesia was discontinued. On the 22nd day of pregnancy and about 2 hours before autopsy, the urinary papilla of the mother was ligated by means of braided silk (Champion No. 6). A few hours before the estimated time for normal parturition, the mother and her fetuses were killed by decapitation (killed at about 21½ days after coitus). Blood emerging during decapitation was allowed to drop into small vials which previously had been moistened with heparin solution. The maternal urine was secured by puncturing the bladder with a hypodermic needle (gauge 27) attached to a 10 cc syringe. The fetal urine was obtained by puncturing the bladder with a micro-pipette prepared from glass tubing and equipped with a small rubber bulb (a "policeman," commonly used in chemical laboratories).[†] By pooling the samples from the fetuses of one mother or, more rarely, from those of several mothers, it was possible to obtain sufficient material for the analyses.

The chemical methods employed were micro-modifications of the following standard

* Aided by grants from the John and Mary R. Markle Foundation and from the medical research funds of the Graduate School.

¹ Wells, L. J., *Anat. Rec.*, 1946, **94**, 504.

² Wells, L. J., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 287.

³ Wells, L. J., *Anat. Rec.*, 1946, **94**, 530.

[†] The pipettes were prepared by Dr. J. Francis Hartmann.

Role of Hemoconcentration in Production of Gastric and Duodenal Ulcer Following Experimental Burns.*

STANLEY R. FRIESEN AND OWEN H. WANGENSTEEN.

From the Department of Surgery, University of Minnesota Medical School, Minneapolis, Minn.

The occurrence of hemoconcentration following extensive burns in man, first noted by Baraduc,¹ is well known. Hemoconcentration was suggested by Kapsinow as the mechanism of the production of Curling's Ulcer.² The role of sepsis in the mechanism of the occurrence of gastric and/or duodenal ulceration following experimental burns has been studied by Hartman.³

The purpose of this study is (1) to evaluate the role of hemoconcentration in the production of ulceration of the gastro-intestinal tract following burns, and (2) to correlate the incidence of hemoconcentration to the incidence of ulceration in dogs subjected to extensive superficial burns, with and without accompanying administration of histamine-in-beeswax mixture, with and without treatment directed to the prevention of hemoconcentration.

When daily intra-muscular injections of histamine-in-beeswax mixture are given to normal dogs, 40 days (or an average of 23 days) are necessary to produce ulcer with regularity.⁴

Method. Healthy dogs, weighing 15 to 36 pounds, anesthetized by intravenous injection of sodium pentobarbital (15 mg per pound), were subjected to 40%, third degree burns by immersion into water at 100°C for 10 to 15 seconds. No evidence of pain or discomfort to the animal was noted. The hair was closely clipped prior to scalding. Blood studies were

* This study is supported in part by the Dr. and Mrs. Harry B. Zimmerman Fund for Surgical Research and the Graduate School of the University of Minnesota, Minneapolis, Minn.

¹ Harkins, H. N., *The Treatment of Burns*, Springfield, Illinois, Charles C. Thomas, publisher, 1942.

² Kapsinow, R., *Southern Med. J.*, 1934, **27**, 500.

³ Hartman, F. W., *Gastroenterology*, 1946, **6**, 130.

⁴ Hay, L. J., Varco, R. L., Code, C. F., and Wangenstein, O. H., *Surg., Gyn. and Obst.*, 1942, **75**, 170.

carried out on all dogs, 3 determinations before the burn for the average of normal values, and serial determinations after the burn. The following determinations were made: Hemoglobin (alkaline hematin method), Hematocrit (Wintrobe method), Erythrocyte Count, Specific Gravity of whole blood and plasma (copper sulfate method of Phillips, Van Slyke, *et al.*), BUN (Karr's urea nitrogen method), Plasma and Blood Volumes (Gibson and Evan's method), and Rectal Temperatures. Blood for these determinations was obtained under oil from the femoral vein before the burn and from the heart following the burn. A number of the dogs received, in addition, daily intramuscular injections of histamine-in-beeswax mixture (30 mg base) prepared after the method of Code and Varco,⁵ the first injection being given 30 minutes prior to the burn. No restriction of food intake was made, except that dogs were fasted for 18 hours before each experiment. Water intake was allowed up to 250 cc per day in all dogs. In some of the animals an attempt was made to prevent the hemoconcentration following burns by slow intravenous administration of human dried plasma into an antecubital vein, regulation of the rate of drip being determined by serial blood studies. Blood pressure determinations were made in 3 dogs by serial or continuous recordings on a kymograph, the carotid artery being cannulated. Dogs were sacrificed 4 hours to 6 days following the burn. Animals in Series II were sacrificed when death was impending or allowed to die. Sections of the stomach, duodenum, liver, lung, brain, kidney, and adrenals were obtained for microscopic studies.

Experiments. Studies were carried out on 30 dogs in 3 series of experiments:

Series I. Fourteen dogs were subjected to 40%, third degree burns accompanied by

⁵ Code, C. F., and Varco, R. L., *Am. J. Physiol.*, 1942, **137**, 225.

stimulus to the fetal kidney. However, a definite elevation of fetal over maternal b.u.n. was observed in only one of 8 observations (10.6 mg%).

Because of the known back diffusion of urea through the tubules a more active clearance of creatinine than of urea might be expected. This proved to be the case.

In addition to these evidences of selective concentration there are the evidences of selective resorption. The exact amount of glucose in the urine was not determinable. However the reducing power of the fetal urine was less than that of the corresponding maternal urine. (After yeast fermentation, reducing substances were no longer detectable in either urine by the method employed). In the light of the concentration of other substances, it can be safely concluded that active resorption of glucose occurred. In this connection it is of interest that histochemical stains show alkaline phosphatase[†] to be already clearly demonstrable in the proximal portion of the secretory tubules of these fetal kidneys.⁹

The resorption of chloride is shown to be more active in the fetus than in the mother.

In further consideration of our observations, the survival of fetal rats taken by Caesarian section indicates that the kidneys are able to function before term,⁹ as in premature infants. But it is a different problem as to whether they actually function during the period when the placental circulation is still intact. The conditions of our experiments were such that the urine we obtained from the bladder of the fetus must have been produced exclusively during this period. This suggests that fetal urine is the

fluid which distends the urinary passages of stillborn infants with hydronephrosis.^{10,11} Makepeace and collaborators¹² and Jacqué¹³ have analyzed fluid from the bladder of fetuses. Although these investigators failed to make it clear that all of the fluid they analyzed was actually produced before the placental circulation had been interrupted, their papers have internal evidence that this was the case.

Our observations indicate that in fetal rats the urine is more or less dilute. To this extent, our findings are in complete accord with those of other investigators.^{12,13} That the fetal urine is actually dilute might be expected in view of the fact that the kidneys of infants are not as effective as those of adults.¹⁴

It is perfectly clear that the work performed by the fetal kidney is not essential for the survival of the fetus and that the placenta (hemochorial) is the essential excretory organ until birth. Recently Potter¹⁵ has reported a series of 20 infants with bilateral agenesis of the kidney.[§]

Finally, the question arises as to whether the production of urine by the human fetus is a factor in such clinical disorders as hydramnios. An experimental approach to this problem, using the rat, is in progress at the University of Minnesota.

Summary. The bladders of fetal rats were allowed to fill after the urogenital papilla had been ligated. The contents of the bladder and the blood of the fetus were analyzed, as were also those of the mother. The observations indicate that the fetal kidney produces urine which is somewhat dilute.

¹³ Jacqué, L., *Arch. Internat. Physiol.*, 1906, **3**, 463.

¹⁴ Young, W. F., McCance, R. A., and Dobbs, R., *Proc. Roy. Soc. Med.*, 1943, **36**, 219.

¹⁵ Potter, E. L., *J. Pediat.*, 1946, **29**, 68.

[†] In fetuses in which hydronephrosis is produced experimentally by ligating the ureter, the phosphatase in their kidneys is being studied by histochemical and microchemical methods.

⁹ Wells, L. J., unpublished observations.

¹⁰ Wells, L. J., and Bell, E. T., *Arch. Path.*, 1946, **42**, 274.

¹¹ Dohrn, *Monatschr. f. Kinderheilkunde*, 1881, **2**, 98.

¹² Makepeace, A. W., Fremont-Smith, F., Dailey, M. E., and Carroll, M. P., *Surg., Gynec. and Obstet.*, 1931, **53**, 635.

[§] In such non-placental forms as chick embryos the nitrogenous wastes are eliminated by the mesonephros and deposited in the allantois.^{16,17} During the first 16 days of incubation, the allantois is the only "urinary bladder."¹⁷

¹⁶ Boyden, E. A., *J. Exp. Zool.*, 1924, **40**, 437.

¹⁷ Fiske, C. H., and Boyden, E. A., *J. Biol. Chem.*, 1926, **70**, 535.

histamine-in-beeswax administration. Blood determinations were carried out in all animals.

Series II. Five dogs were subjected to 40% third degree burns alone, blood determinations being carried out in all dogs.

Series III. Six dogs were subjected to 40% third degree burns accompanied by histamine-in-beeswax administration, and attempts to prevent the hemoconcentration were carried out by intravenous infusion of plasma. One dog (No. 41) received, in addition, 5% glucose in normal saline, and another dog (No. 49) received 5% glucose in normal saline alone. As an additional control, one dog (No. 48), not subjected to a burn, received histamine-in-beeswax, was anesthetized, and received a comparable amount of plasma by intravenous infusion. Four additional dogs received daily intramuscular injections of histamine-in-beeswax for 5 successive days, and were sacrificed at the end of this period.

Results. Series I. (See Table I). Twelve of the 14 dogs subjected to 40% burns accompanied by histamine administration developed increased concentration of the blood. All 12 dogs demonstrated marked congestion of the mucous membrane of the stomach and duodenum. Nine of these 12 dogs were sacrificed 24 hours or more after the burn and showed definitive ulcers, 5 bleeding and 2 perforated. Three of the 12 dogs were sacrificed at 4, 6, and 18 hours after the burn, the latter showing in addition to the congestion, marked diffuse bleeding from the surface of the gastric and duodenal mucosa. The 2 dogs which failed to develop increased concentration of the blood demonstrated no gastric or duodenal pathology in one and 4 days.

Series II. (See Table II). Four of the 5 dogs subjected to 40% burns without administration of histamine-in-beeswax developed increased concentration of the blood and demonstrated congestion of the gastrointestinal mucosa in 2 to 6 days. Two of these dogs showed, in addition to congestion, bleeding petechial gastric ulcers, and antral bleeding points with hemorrhagic gastritis and duodenitis. One dog failed to develop increased concentration of the blood and demonstrated no gastric or duodenal pathology in 4 days.

TABLE II.
Dogs Subjected to 40% Burns—No Histamine Administration—Not Treated with Intravenous Infusion.

Dog No.	Wt in lb	Burn % sec	No. of hist. injections	Hematocrit change		Results	Remarks
				Initial	Final		
26	26	40 10	0	50	56	Bleeding petechial gastric ulcers; congestion	Sacrificed 6th day
27	31	40 10	0	45	57	Hemorrhagic gastritis duodenitis; antral bleeding pts.	Died 2nd day
28	15	40 10	0	50	60	Marked congestion	" 4th "
29	24	40 15	0	48	59	Essentially negative	Sacrificed 5th day
30	30	40 15	0	45	56	Congestion only	Died 5th day

TABLE I.
Dogs Subjected to 40% Burn Accompanied by Daily Administration of Histamine-in-Beeswax (30 mg Base), Not Treated with Intravenous Infusion.

Dog No.	Wt in lb	Burn		No. of hist. injections	Hematocrit change		Results	Remarks
		%	sec		Initial	Final		
23	29	40	10	4	40	41	No congestion, erosion, or ulcer	Died—anoxia
24	32	40	10	5	44	35	Congestion; duodenal ulcers, one perforated	Died—peritonitis
25	22	40	10	3	42	53	Congestion; pyloric and duodenal ulcers	Died the third day
36	17	40	15	1	49	56	Congestion; small pyloric ulcer; many bleeding points	Sacrificed, 24 hr
37	22	40	15	2	43	65	Congestion; bleeding pyloric ulcer; two large duodenal ulcers	" 48 "
38	24	40	15	2	50	61	Congestion; pyloric-duodenal ulcer; bleeding small fundic ulcers	Died second day—melena
39	22	40	15	2	30	41	Congestion; large duodenal ulcers, one perforated; fundic ulcers	Died—peritonitis
40	24	40	15	2	50	55	Congestion; three large duodenal ulcers; one large gastric ulcer; many small bleeding ulcers	Sacrificed—melena
42	15	40	15	1	40	57	Congestion; marked bleeding in stomach and duodenum	" 18 hr
44	20	40	15	1	49	72	Intense congestion	" 4 "
45	26	40	15	3	47	36	Congestion; bleeding antral ulcer	Died third day
50	22	40	10	1	42	42	No congestion, erosion, or ulcer	Sacrificed, 24 hr
51	36	40	10	1	40	54	Congestion only	" 6 "
52	31	40	15	1	45	53	Congestion; large duodenal ulcer; many petechial gastric ulcers	" 24 "

tration and mucosal pathology following the burns may be attributable to the fact that those animals received a 40% 10-second burn, a less severe burn than the majority of the animals in this study received. Serial blood pressure determinations were made in 3 burned dogs, continuously anesthetized, which developed hemoconcentration, and no readings of shock level were observed. Animals were usually awake and moving about after 4 hours following the burn.

It is interesting to note that superficial necrosis of the gastric mucosa was present upon microscopic examination in 2 dogs, both sacrificed, one after marked hemodilution due to overtreatment, the other after marked hemoconcentration and congestion of the mucosa. Microscopically, the vascular spaces of the mucosa of the overtreated animal were conspicuously devoid of blood, while those of the untreated animal were markedly dilated and engorged with blood with extravasa-

tion and necrosis. Both conditions result in mucosal ischemia, susceptible to the erosive action of gastric acid-peptic secretions.

Conclusions. Evidence is presented to show that increased concentration of the blood following burns is an important factor in the occurrence of gastro-duodenal ulcer after experimental burns. (The occurrence of gastro-intestinal congestion, erosion and/or ulcer in burns is directly related to the occurrence of hemoconcentration).

Moreover, gastro-intestinal abnormality following burns, even when accompanied by histamine administration, may be prevented by avoidance of the hemoconcentration of burns by proper therapy.

The incidence of gastric and/or duodenal ulceration provoked by hemoconcentration in burns is markedly increased when histamine-in-beeswax administration accompanies the burn.

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Comparative Utilization of Raw and Autoclaved Soy Bean Protein by the Human.

JESSICA H. LEWIS AND F. H. L. TAYLOR.

(With the technical assistance of M. A. Adams and L. J. Harrison.)

From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston.

Osborne and Mendel¹ in 1917 noted that rats fed raw soy bean flour grew more slowly than a control group which received the same flour after it had been autoclaved. Hayward, Steenbock and Bohstedt² confirmed these investigations and suggested that this effect was not due to a difference in palata-

bility but to a deficiency in the raw flour. Later investigations by Hayward and Hafner,³ Almquist, Mecchi, Kratzer and Grau⁴ and Johnson, Parsons, Steenbock⁵ suggested that this increase in biological activity produced by autoclaving raw soy bean flour was due to a rise in the level or increase in the availability of sulfur bearing amino acids.

That the autoclaved soy bean protein has a high biological value similar to egg white and liver protein has been shown by Cahill, Schroeder and Smith⁶ from a study of ni-

¹ Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, **32**, 369.

² Hayward, J. W., Steenbock, H., and Bohstedt, G., *J. Nutrition*, 1936, **11**, 219.

³ Hayward, J. W., and Hafner, F. H., *Poultry Science*, 1941, **20**, 139.

⁴ Almquist, H. J., Mecchi, E., Kratzer, F. H., and Grau, C. R., *J. Nutrition*, 1936, **63**, 277.

⁵ Johnson, Y. M., Parsons, H. T., and Steenbock, H., *J. Nutrition*, 1939, **18**, 423.

⁶ Cahill, W. M., Schroeder, L. J., and Smith, A. H., *J. Nutrition*, 1944, **28**, 269.

⁷ McNaught, J. B., Scott, V. C., Woods, F. M., and Whipple, G. H., *J. Exp. Med.*, 1936, **63**, 277.

TABLE III.
Dogs Subjected to 40% Burn, Accompanied by Histamine-in-Beeswax Administration, Treated by Intravenous Infusion.

Dog No.	Wt in lb	Burn		No. of hist. injections	Concentration of blood	Results	Remarks
		%	see				
43	21	40	15	1	Maintained at normal values	No congestion, erosion, or ulcer	Sacrificed after 18 hr
49	32	40	15	1	" " "	" " "	" " 24 "
53	35	40	15	1	" " "	" " "	" " 24 "
46	18	40	15	2	Normal except for 18 hr of hemoconcentration	Congestion, lesser curvature ulcers	" " 24 "
47	19	40	15	2	Normal except for 18 hr of hemoconcentration	Congestion, perforated duodenal ulcers	Died, peritonitis, 54 hr
41	22	40	15	1	Overtreated—hemolysis	Pallor, submucosal and mucosal hemorrhage and erosion of stomach	Died 16 hr

Series III. (See Table III). Of the 6 dogs subjected to 40% burns accompanied by histamine-in-beeswax administration in which an attempt at prevention of the hemoconcentration in burns was made, 3 dogs were well controlled throughout the entire duration of the experiment; 2 dogs, partially controlled; and one dog, overtreated. The 3 dogs in which blood concentration values were maintained at normal levels demonstrated no gastric or duodenal pathology when sacrificed in 18 to 24 hours. The 2 dogs in which normal blood concentration values were maintained except for a period of 18 hours out of a total duration of 54 hours, demonstrated gastro-intestinal congestion, with lesser curvature ulcers and perforated duodenal ulcer, respectively. One dog was overtreated in the first few hours of the experiment so that marked hemodilution occurred. Death occurred in this animal in 16 hours, presenting pallor of the stomach with several areas of mucosal and submucosal hemorrhage and erosions.

Microscopic examination of the viscera of the animals developing hemoconcentration showed marked vascular congestion; of the animals in which hemoconcentration did not take place or was prevented, no significant findings were noted.

Four dogs receiving daily intramuscular injections of histamine-in-beeswax for 5 days developed no hemoconcentration and no gastro-intestinal abnormality.

Discussion. It is apparent that there exists a close correlation between increased concentration of the blood and gastro-intestinal abnormality following burns. That hemoconcentration, with its resultant congestion, renders the gastro-duodenal mucosa more susceptible to the action of gastric acid-peptic juice is evident in the marked increase in the incidence of ulceration when histamine-in-beeswax administration accompanies the burn. It is shown, in addition, that prevention of the occurrence of hemoconcentration following severe burns portends a normal gastro-intestinal tract, even when histamine-in-beeswax is administered and animals are sacrificed in less than 5 days. Failure of 3 dogs in Series I and II to develop hemoconcentration

of an aqueous extract of raw soy bean. This was also shown by Kunitz¹² who later isolated this trypsin inhibitor in crystalline form.¹³

Tagnon and Soulier¹⁴ have shown that this trypsin inhibitor acts *in vitro* as an anti-coagulant of whole blood and plasma, suggesting that it may also inhibit the plasma enzyme associated with blood coagulation.

In view of the current widespread use of soy bean flour as a human food, and because of the observations made in animals, the investigations reported here were undertaken to compare the utilization of raw and autoclaved soy bean flour in humans.

Methods and Materials. Two cooperative, well nourished patients, one with a diagnosis of mild osteoarthritis; the other a hemophilic without hemorrhagic manifestations, were chosen as subjects. Both patients were allowed unrestricted activity about the ward and their caloric requirements were estimated at 2500 calories. The basic diet was, therefore, planned to afford 2500 calories daily and provided a low protein intake of 25 g from mixed sources, about 75% of which was of vegetable origin. After an initial control period of 8 days, during which the subjects received only the basic diet, 188 g of raw soy bean flour containing 75 g of protein was substituted for an equal caloric value of carbohydrate in the basic diet. The soy bean flour was given as a liquid supplement dissolved in water to a volume of one liter. After this 9-day period on raw soy bean flour the subjects returned to the basic diet for 6 days. This was followed by another 9-day trial period during which the subjects received 188 g of soy bean flour previously autoclaved for one hour at 15 lb pressure.

Nitrogen balance and body weights were followed throughout the study period.

Urine nitrogen determinations were done by micro-Kjeldahl method on aliquot of 24-hour specimens, and stool nitrogens were de-

termined by macro-Kjeldahl method on 3-day pooled specimens.

Results. Fig. 1 and 2 show the caloric intake and nitrogen balances of these 2 patients. The average daily nitrogen retention of patient J.B. was 6.7 g on raw soy bean flour and 8.5 g on the autoclaved material. Patient R.W. showed similar results with an average daily nitrogen retention for the first period of 4.1 g and for the second period of 5.4 g. In each of these subjects the nitrogen retention is about 20% greater when the autoclaved material is used instead of the raw material. Increase in stool nitrogen does not account for this difference as subject J.B. showed an average daily stool nitrogen of 1.3 g during the first period and 1.1 g during the second period, while subject R.W. showed 1.6 g in the first period and 1.8 g in the second period. There was no significant change in body weight in either patient.

The soy bean supplements were not disagreeable in taste. Both patients complained of flatulence during both of the trial periods. There were no other gastro-intestinal symptoms.

Discussion. Both the raw and autoclaved soy bean protein support positive nitrogen balance in adult humans. Retention of nitrogen is about 20% greater during the administration of the autoclaved material than with the raw material. The cause of this greater biological activity is not clear from these investigations. There is no increase in excretion of nitrogen in the stool following intake of raw material, suggesting that intestinal protein digestion has progressed far enough to produce absorbable nitrogen products. Whether all these products absorbed from nonautoclaved soy bean flour are capable of entering the metabolic pool requires further study.

Conclusions. 1. Both raw and autoclaved soy bean protein support positive nitrogen balance in the adult human. 2. Nitrogen retention is about 20% greater with the autoclaved material than with the raw material.

¹⁴ Tagnon, H. J., and Soulier, J. P., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61**, 440.

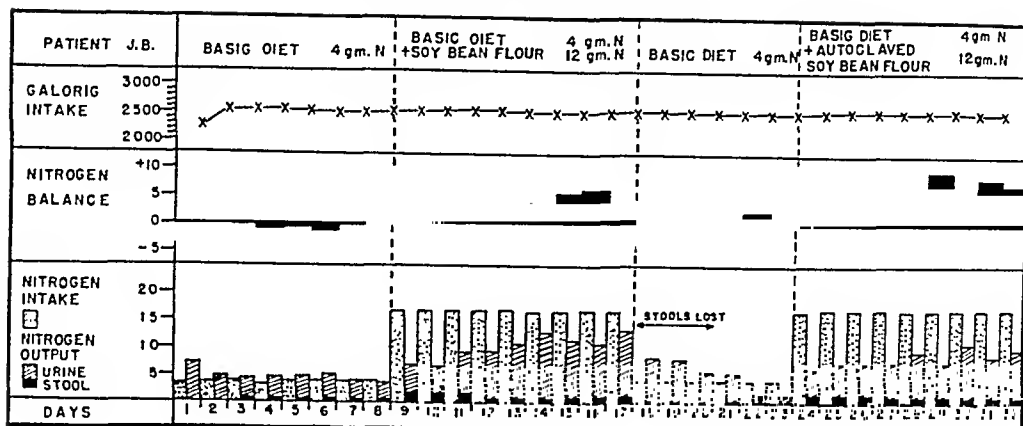


FIG. 1.
Nitrogen balance in Patient J. B.

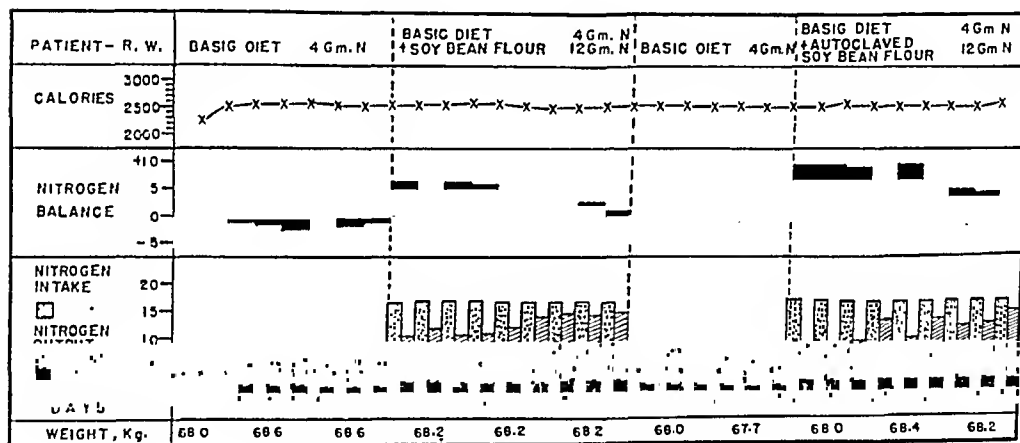


FIG. 2.
Nitrogen balance in Patient R. W.

trogen balance in humans and by McNaught, Scott, Woods and Whipple⁷ from a study of plasma protein regeneration in dogs.

Recently another explanation has been offered for the apparent lower biological activity of raw soy bean protein. Ham and Sandstedt⁸ isolated a trypsin-inhibiting substance from the unheated soy bean flour which they felt was identical with its growth-retarding action. In later studies, Ham, Sandstedt and Mussehl⁹ showed that this

trypsin-inhibiting substance isolated from soy bean flour had a retarding effect upon the normal growth of chicks; and, by *in vitro* test they showed that the proteolytic activity in the small intestine of the chick was greatly inhibited. Similar results were obtained by Klose, Hill and Fevold¹⁰ in growing rats.

Bowman¹¹ demonstrated the inhibition of the tryptic digestion of casein by a fraction

⁸ Ham, W. E., and Sandstedt, R. M., *J. Biol. Chem.*, 1944, **154**, 505.

⁹ Ham, W. E., Sandstedt, R. M., and Mussehl, F. E., *J. Biol. Chem.*, 1945, **161**, 635.

¹⁰ Klose, A. A., Hill, B., and Fevold, H. L., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 10.

¹¹ Bowman, D. E., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 139.

¹² Kunitz, M., *Science*, 1945, **101**, 668.

¹³ Kunitz, M., *J. Gen. Physiol.*, 1946, **29**, 149.

of an aqueous extract of raw soy bean. This was also shown by Kunitz¹² who later isolated this trypsin inhibitor in crystalline form.¹³

Tagnon and Soulier¹⁴ have shown that this trypsin inhibitor acts *in vitro* as an anticoagulant of whole blood and plasma, suggesting that it may also inhibit the plasma enzyme associated with blood coagulation.

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Discussion. Both the raw and autoclaved soy bean protein support positive nitrogen balance in adult humans. Retention of nitrogen is about 20% greater during the administration of the autoclaved material than with the raw material. The cause of this greater biological activity is not clear from these investigations. There is no increase in excretion of nitrogen in the stool following intake of raw material, suggesting that intestinal protein digestion has progressed far enough to produce absorbable nitrogen products. Whether all these products absorbed from nonautoclaved soy bean flour are capable of entering the metabolic pool requires further study.

Conclusions. 1. Both raw and autoclaved soy bean protein support positive nitrogen balance in the adult human. 2. Nitrogen retention is about 20% greater with the autoclaved material than with the raw material.

¹⁴ Tagnon, H. J., and Soulier, J. P., *Proc. Soc. Exp. Biol. and Med.*, 1949, **61**, 449.

Susceptibility of the Bear to Fox Encephalitis.

C. S. STULBERG AND R. G. GREEN.

From the Department of Bacteriology and Immunology, University of Minnesota, Minneapolis.

Fox encephalitis¹ is a common disease of the North American silver fox and has been identified on fox ranches throughout the United States. Red foxes, dogs, and coyotes² are also highly susceptible. Although not generally recognized clinically, fox encephalitis frequently appears to occur as a natural disease in dogs.³ The gray fox,⁴ which is related to the South American foxes and dogs, and the raccoon⁵ are only slightly susceptible to experimental infection.

Inoculation of the anterior chamber of the eye affords a direct and easy method of identifying the fox encephalitis virus and of establishing susceptibility to infection.⁶ In foxes and dogs such inoculation results in an acute systemic infection and encephalitis with a high mortality. In the inoculated eye the virus damages the single layer of endothelial cells which line the posterior surface of the cornea, resulting in a grayish corneal opacity. The characteristic intranuclear inclusions produced by the virus can be seen in these endothelial cells. Only the injected eye will show the opacity. Injection of the virus into the anterior chambers of the eyes of the less susceptible raccoon and gray fox produces regularly the corneal opacity, but these animals otherwise generally remain well. The cat appears immune to the general infection, but with young kittens⁷ intra-ocular injection

either is negative or results in incomplete opacity. The eye inoculation has been found negative in rabbits.⁷

We have now been able to show that the zoologic range covered by the virus of fox encephalitis also includes the black bear, *Euarctos americanus*. Three specimens of this animal received at our laboratories have been tested for susceptibility to the virus.

Fox encephalitis virus, preserved as 20% homogenized fox brain in 50% neutral glycerin, was prepared in serial, ten-fold dilutions in Ringer's solution. To determine the minimal infective dose that would produce corneal opacity in foxes, 0.1 cc of each dilution was injected into the anterior chambers of the eyes of red fox pups. Since we desired only to approximate the titer of the virus, only one eye was inoculated per dilution, and the dilutions were carried out to 10⁻⁶. The virus proved active at a dilution of 10⁻⁶.

A young, wild black bear was received in the laboratory in rather poor condition. It was underweight, extremely irritable, and did not eat well. This animal was anesthetized with ether and was injected intra-ocularly with 0.1 cc of a 10⁻² dilution of virus in the right eye and 0.1 cc of a 10⁻⁴ dilution in the left eye. Within 3 days the bear developed a complete opacity in its right eye and a partial opacity in its left eye. On the 4th day after inoculation the animal was found dead, without previous symptoms or autopsy findings of fox encephalitis. However, a smear of the endothelial cells of the posterior surface of the cornea revealed the characteristic fox encephalitis inclusion bodies. Aqueous humor removed from the eyes was cultured on blood agar and was found free of bacteria.

The intra-ocular test was repeated on a second young black bear. This animal although somewhat underweight otherwise appeared normal. The virus, diluted with saline, was inoculated into the anterior chambers of the left and right eyes, with 0.2 cc of 10⁻² and 10⁻⁴ dilutions respectively as the

¹ Green, R. G., Ziegler, N. R., Green, B. B., and Dewey, E. T., *Am. J. Hyg.*, 1930, **12**, 109.

² Green, R. G., Ziegler, N. R., Carlson, W. E., Shillinger, J. E., Tyler, S. H., and Dewey, E. T., *Am. J. Hyg.*, 1934, **19**, 343.

³ Green, R. G., and Shillinger, J. E., *Am. J. Hyg.*, 1934, **19**, 362.

⁴ Green, R. G., unpublished data.

⁵ Green, R. G., Evans, C. A., and Yanamura, H. Y., *Proc. Soc. Exp. Biol. and Med.*, 1943, **53**, 186.

⁶ Evans, C. A., Yanamura, H. Y., and Green, R. G., *Proc. Soc. Exp. Biol. and Med.*, 1943, **53**, 183.

⁷ Evans, C. A., and Green, R. G., *Staff Meeting Bulletin, Hospitals of the University of Minnesota*, 1945, **16**, 142.

inoculum. This animal showed no sign of susceptibility to the virus until the 10th day after inoculation. When partial corneal opacity of the left eye appeared, becoming complete 2 days later. No further observations were possible since it became necessary at that time to kill the bear. However, the animal did exhibit intranuclear inclusions of fox encephalitis virus in the corneal endothelium. The aqueous humor was bacteriologically sterile.

A third black bear, which was half grown,

was inoculated intramuscularly with 5 cc of 20% virus but did not develop observable symptoms.

Conclusions. The intra-ocular inoculation of two bears and the intramuscular injection of a third with fox encephalitis virus indicate that the black bear (*Euarctos americanus*) is only slightly susceptible to this virus infection. Since the bear, like the raccoon, is an offshoot of the canines, the pathogenic properties of fox encephalitis virus derived from foxes seem sharply confined to the canine family.

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Two Related Salmonella Types: *S. luciana* and *S. marseille*.*

ALICE B. MORAN, P. R. EDWARDS, AND D. W. BRUNER.

From the Department of Animal Pathology, Kentucky Agricultural Experiment Station, Lexington, Ky.

A. *Salmonella luciana* was isolated by Mrs. Mildred Galton from the feces of a normal food handler. The organism fermented glucose, xylose, arabinose, rhamnose, maltose, cellobiose, trehalose, mannitol, sorbitol, and dulcitol with production of acid and gas. Lactose, sucrose, inositol, and salicin were not fermented. The culture utilized *d*, *l*, and *i*-tartrate, mucate, and citrate. Hydrogen sulfide was produced but indol was not formed nor was gelatin liquefied.

The organism was agglutinated to the titre of, and absorbed all agglutinins from *S. aberdeen* O serum. Its somatic antigens are XI.

S. luciana was diphasic and the H antigens of phase I were identical with the H antigens of *S. paratyphi* A (a). The antigens of phase 2 were identical with phase 2 of *S. glostrup* (e.n.z₁₅...). The antigenic formula of *S. luciana* is XI:a-e.n.z₁₅...

B. *Salmonella marseille* was isolated from the stools of a person affected with gastro-

enteritis, by Captain W. B. Sutton of the 4th Medical Laboratory, U. S. Army. Its biochemical properties differed from those of *S. luciana* only in that it produced acid from raffinose after 9 days incubation and it did not utilize *i*-tartrate.

The somatic antigens of *S. marseille* were identical with those of *S. aberdeen* (XI). The culture was diphasic and phase 1 resembled that of *S. paratyphi* A (a) although when used to absorb *S. paratyphi* A serum a slight residue of agglutinins remained for *S. paratyphi* A, *S. luciana*, and *S. loma-linda*.

Phase 2 of *S. marseille* was agglutinated by serums for all the nonspecific phases of the Kauffmann-White classification. When tested with serums for single factors 2, 3, 5, 6, 7, 10, and 11 it was agglutinated only by serums for factors 3 and 5. In absorption tests phase 2 of *S. marseille* removed all agglutinins from serum derived from phase 2 of *S. thompson* (1.5...). The diagnostic formula of *S. marseille* is XI:a-1.5...

Summary. Two new Salmonella types were described. *S. luciana* (XI:a-e.n.z₁₅...) was recovered from the feces of a normal food handler while *S. marseille* (XI:a-1.5...) was isolated from the stools of a patient affected with gastro-enteritis.

* The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director. It was supported in part by a research grant from the U. S. Public Health Service.

Plasma Protein Concentrations and Organ Weights of Rats as Related to a High Protein Diet.*

JAMES H. LEATHEM.

From the Bureau of Biological Research and the Department of Zoology, Rutgers University, New Brunswick, N.J.

Adrenal hypertrophy resulting in an increased functional level of the cortex has been observed in rats fed a high protein diet and may be related to an increased rate of protein catabolism¹ but not all investigators have observed an increase in adrenal weight in either rats^{2,3} or mice⁴ eating a diet high in protein content. Furthermore, no change in plasma protein concentrations was observed in rats fed a 78% casein diet.⁵ It was presumed that a change in functional activity of the adrenal cortex would be reflected in plasma protein levels since an important relationship between the adrenal cortex and plasma protein concentrations is known to exist.^{5,6}

Ingle⁷ reinvestigated the high protein diet-adrenal weight problem in both young and adult rats fed a diet of 67% protein and found the adrenals to be unchanged, but did observe a modest adrenal weight increase when dietary protein was increased to 80%. The primary protein sources used by Ingle were casein (Labco) and lactalbumin. Since a difference in the protein source itself can influence the experimental results as has been effectively demonstrated in adrenalectomized rats given 2 types of casein,⁸ the

current investigation involving a restudy of the adrenals in rats fed a high protein diet was undertaken and plasma protein levels were again used as a possible means of detecting an increased functional activity of the adrenal cortex.

Adult male rats 130 to 157 days of age were used. The diets fed were Purina fox chow as the control diet (22.8% protein) and the high protein diets which were composed of 78% casein (Merck) or lactalbumin (Borden, lot No. 15-42), 10% brewers yeast (Mead-Johnson), 5% Wesson's salt mixture, 5% Mazola and 2% cod liver oil (Squibb). The diet including casein simulated the Casein A diet of Tepperman *et al.*¹ Lactalbumin was chosen as another primary protein source because the nitrogen balance index for this protein is higher than that of casein.⁹

The experimental period was 20-27 days in duration and during the first 20 days food intake ranged between 280-291 g total per rat fed the high protein diets as compared with a 336 g total per rat fed fox chow. The rats were bled by cardiac puncture under light anesthesia at the conclusion of the experiment and the plasma analyzed for NPN, total protein, albumin and globulin by methods reported previously.¹⁰ Each rat was autopsied and fresh weight of the carefully dissected pituitary, adrenal, thyroid, testis, seminal vesicle, kidney and liver was recorded.

Adult rats fed a high casein diet gained an average of 16 g in body weight as compared with a 28 g gain by rats eating fox chow. When lactalbumin was substituted for casein an average gain of only 4 g in body weight was recorded whereas the littermate controls

* Supported by the Protein Metabolism Fund of the Bureau of Biological Research, Rutgers University.

¹ Tepperman, J., Engel, F. L., and Long, C. N. H., *Endocrinology*, 1943, **32**, 403.

² Ingle, D. J., Ginther, G. B., and Nezamis, J., *Endocrinology*, 1943, **32**, 410.

³ Leatham, J. H., *Endocrinology*, 1945, **37**, 157.

⁴ Benua, R. S., and Howard, E., *Endocrinology*, 1945, **36**, 170.

⁵ Levin, L., and Leatham, J. H., *Am. J. Physiol.*, 1942, **136**, 306.

⁶ White, A., and Dougherty, T. F., *Endocrinology*, 1945, **36**, 207.

⁷ Ingle, D. J., *Endocrinology*, 1945, **37**, 7.

⁸ Segaloff, A., *Endocrinology*, 1946, **38**, 26.

⁹ Allison, J. B., Anderson, J. A., and Seeley, R. D., *Ann. N. Y. Acad. Sci.*, 1946, **47**, 245.

¹⁰ Leatham, J. H., *Endocrinology*, 1945, **36**, 98.

TABLE I.
Influence of Diet on Organ Weight of Adult Male Rats.

Diet (No. of rats)	Casein 78% (14)	Fox chow (14)	Lactalbumin 78% (12)	Fox chow (12)
Avg organ weight—mg.				
Adrenal	34.8 ± 2.2*	28.4 ± 1.4	34.8 ± 2.2	28.6 ± 2.0
Thyroid	23.6 ± 0.7	23.0 ± 0.5	22.8 ± 0.9	22.6 ± 0.4
Kidney	3248.0 ± 49	2632.0 ± 47	2962.0 ± 123	2811.0 ± 157
Liver	13579.0 ± 289	12303.0 ± 302	12595.0 ± 655	12649.0 ± 341
Avg organ weight—mg/100 g body weight.				
Adrenal	10.7 ± 0.7	8.7 ± 0.5	11.2 ± 0.7	8.8 ± 0.6
Thyroid	7.3 ± 0.2	7.0 ± 0.2	7.4 ± 0.3	7.3 ± 0.1
Kidney	1008.0 ± 15	818.0 ± 14	959.0 ± 42	787.0 ± 57
Liver	4192.0 ± 83	3786.0 ± 94	4009.0 ± 220	3870.0 ± 97

$$s_e = \sqrt{\frac{\sum d^2}{n(n-1)}}$$

TABLE II.
Influence of Dietary Protein on the Plasma Protein Levels of Male Rats.

No. of rats	Diet	Body wt start-end, g	Hemato-crit, %	Non-protein N, mg/100 cc	Total protein, g/100 cc	Albumin, g/100 cc	Globulin, g/100 cc
Rats 130-157 days old.							
14	Casein 78%	307-323	48.5 ± 0.7	76 ± 1.8	6.08 ± 0.15	3.06 ± 0.9	3.02 ± 0.08
14	Fox chow	296-324	47.6 ± 0.6	64 ± 2.0	6.13 ± 0.11	3.16 ± 0.10	2.96 ± 0.10
12	Lactalbumin 78%	309-313	47.5 ± 0.7	73 ± 2.1	6.01 ± 0.14	3.06 ± 0.14	2.95 ± 0.16
12	Fox chow	307-326	47.4 ± 0.3	60 ± 1.1	6.23 ± 0.12	3.29 ± 0.10	2.97 ± 0.16
Rats 45-49 days old.							
8	Lactalbumin 78%	142-204	43.3 ± 0.5	80 ± 2.0	5.40 ± 0.19	3.20 ± 0.10	2.20 ± 0.17
8	Fox chow	144-227	44.4 ± 0.4	51 ± 1.2	5.87 ± 0.18	3.45 ± 0.09	2.42 ± 0.20

TABLE III.
Influence of Diet on Organ Weight of Young Male Rats.

Diet (No. of rats)	Lactalbumin 78% (8)		Fox chow (8)		Lactalbumin 78% (8)		Fox chow (8)	
	Avg organ wt, mg				Avg organ wt, mg/100 g B.W.			
Adrenal	33.9	± 3.2	27.2	± 2.3	15.8	± 0.9	12.1	± 0.8
Thyroid	17.2	± 2.1	16.4	± 1.4	8.3	± 0.7	7.5	± 0.6
Kidney	2412.0	± 161	1987.0	± 63	1161.0	± 74	875.0	± 30
Liver	10595.0	± 663	9770.0	± 538	5127.0	± 340	4315.0	± 261

on stock diet gained 19 g during the same 20-day period.

It is apparent from the data in Table I that the adrenals from rats fed a high protein diet of either casein or lactalbumin had a tendency to be somewhat heavier than the adrenals from rats fed fox chow but the differences are not striking. Kidney and liver weights too were greater in rats fed 78% casein but not in those fed 78% lactalbumin. This seeming difference in results between the 2 high protein diets and kidney weight is shown to be due to body weight differences (Table I). Although it has been

reported that thyroid hypertrophy can be induced by excess dietary protein, no indication of a change in thyroid weight was observed. Weights of the pituitary, testis and seminal vesicles were not influenced by diet.

Plasma protein concentrations failed to reveal any evidence that the functional level of the adrenal cortex was altered by a diet containing 78% casein or lactalbumin. The data in Table II reveal that albumin and globulin concentrations and consequently total plasma protein levels were not significantly influenced by the diet. Non-protein-nitrogen, however, was elevated when excess

protein was fed.

The possibility that young rats might exhibit a more pronounced response to a diet containing 78% lactalbumin than did the adults prompted an investigation of this point. Eight rats, 45-49 days old, were fed the 78% lactalbumin diet for an average of 23 days and the data were compared with littermate controls fed fox chow. Despite *ad libitum* feeding, the rats consuming the high protein diet gained an average of only 62 g as compared to the average 83 gain exhibited by rats eating fox chow (Table II). Despite the retardation in body weight gain, the adrenal, kidney and liver weights from rats fed a 78% lactalbumin diet exceeded those from rats fed stock diet. These organ weight differences are more apparent when organ weight is considered in terms of 100 g body weight (Table III). The weight of the thyroid, testis, seminal vesicles and pituitary was not influenced.

Examination of the plasma protein concentrations for total protein, albumin and globulin suggested that rats fed fox chow had the higher plasma protein levels but the differences are not clearly significant. NPN, however, was significantly higher in rats fed the high protein diet.

Since liver protein can be readily influenced by a dietary deficiency in protein it was of interest to examine the livers from some of these young rats under the opposite conditions or that of protein excess. The livers

from 4 pairs of rats were dried to constant weight at 95°C and analyzed for total protein. Water content of the livers from rats fed 78% lactalbumin was 69.9% and from rats fed fox chow was 70.0%. Liver protein was found to be greater in the lactalbumin-fed rats than in stock-diet animals. Following the feeding of the high protein diet the livers contained an average 1.15 (0.98-1.26) g protein/100 g body weight as compared with an average 0.83 (0.76-0.89) g protein/100 g body weight. Furthermore, the percentage protein of the dry liver increased from 64.0% in rats fed fox chow to 72.3% in rats fed the high lactalbumin diet and therefore the increased liver protein was not alone due to increase in liver size.

Summary. The effect of a high protein diet (78% casein or lactalbumin) was studied in male rats. The rate of body weight increase was retarded over a 3-week period by a 78% lactalbumin diet in young and adult rats. A modest increase in adrenal weight was observed but no indication of increased functional activity was suggested by alterations in plasma albumin or globulin concentrations since the plasma protein levels remained unchanged. Non-protein nitrogen was elevated by the excess dietary protein. Thyroid, pituitary, testis and seminal vesicle weights remained normal but kidney and liver weights were increased. Liver protein was increased in young rats fed the high lactalbumin diet.

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Pyribenzamine Aerosol Inhalation and its Influence on Histamine Poisoning and Anaphylaxis.

R. L. MAYER, D. BROUSSEAU, AND P. C. EISMAN. (Introduced by F. F. Yonkman.)

From Ciba Pharmaceutical Products, Inc., Summit, N.J.

The lethal dose of histamine for guinea pigs by intravenous injection is 0.3 to 0.4 mg/kg body weight,¹ but the amounts required

to kill guinea pigs when introduced into the lungs by aerosol inhalation are fractions of these doses.^{2,3} Indeed, many guinea pigs breathing air containing less than 0.1 γ his-

¹ Guggenheim, M., *Die Biogenen Amine*, Basle, 1940.

² Halpern, B. N., *Arch. internat. de pharmacodyn.*

et de Therap., 1942, **68**, 339.

³ Mayer, R. L., *J. Allergy*, 1946, **17**, 153.

tamine per ml die within 2 to 5 minutes. The high activity of histamine introduced directly into the lungs is explained by two facts, namely, that the lungs of guinea pigs contain tissues whose cells are exceedingly susceptible to this substance, and that the marked response of these cells in histamine poisoning is the primary cause of death.

Substances such as Pyribenzamine (PBZ) for example, which specifically counteract histamine, are believed to prevent histamine from acting upon organs susceptible to histamine. According to a theory accepted by many investigators, these antihistaminic substances compete with histamine, blocking the receptive sites within the susceptible cells which normally respond readily to histamine. If this theory is correct it would be expected that a specific antihistaminic substance, like PBZ, introduced directly into the lungs by aerosol inhalation would provide considerably more protection against subsequent histamine poisoning than when injected or given by mouth. Moreover, it might also be found that PBZ, as an aerosol, would counteract histamine or protect against anaphylaxis in such sufficiently small doses that side effects, common to the usual therapeutic administration, could be avoided.

Experiments with PBZ aerosol could therefore provide us with valuable information concerning the mechanism of action of antihistaminic substances. On the other hand, it seemed to us probable that a comparison of the effect of PBZ aerosols upon histamine poisoning and anaphylaxis might lead to further insight into the relationship between histamine poisoning and anaphylaxis. This question is especially important since there are many authors who consider histamine to be identical with the anaphylactic poison, while others reject the histamine theory of anaphylaxis.

In the following experiments we therefore investigated in guinea pigs the influence of PBZ aerosols upon the tolerance toward histamine and upon active and passive anaphylaxis.

Methods. 173 guinea pigs weighing from 150 to 200 g were subjected to inhalation of PBZ aerosols in the same manner as previ-

ously described for histamine aerosols.²⁻⁶ In the various experiments performed, we vaporized solutions containing 0.5, 1, and 2% PBZ (hydrochloride), the air flow at the outlet of the vaporizers being maintained at approximately 20 liters per minute.

The influence of the aerosol inhalation upon histamine intoxication and anaphylaxis was tested as follows: In the first series of experiments, the duration of the PBZ aerosol treatment was varied and the influence of each time-treatment upon the resistance of the animals to histamine was measured. In the second series we kept the duration of aerosol-treatment constant and determined the duration of protection to histamine. In both series histamine was injected intracardially as histamine phosphate. In the third and fourth series of experiments we tested in an identical way the influence of PBZ aerosol inhalation upon active and passive anaphylaxis. Active anaphylaxis was produced with horse serum by the usual technic, the intracardial "challenge" injections being made three weeks after sensitization, and passive sensitization by injection with anti-horse rabbit serum, followed by the "challenge" injection 24 hours later.

Results. Histamine Shock. I. Histamine Intoxication. In preliminary trials, the sensitivity of guinea pigs to the histamine used for these experiments was determined. The minimal toxic dose producing more than 50% deaths was found to be between 0.7 to 0.8 mg histamine phosphate per kg, corresponding to 0.25 to 0.29 mg of histamine respectively. The MLD₅₀ was about 25% higher in the present experiments than in our earlier work,³ a fact which may have been due to variation among the histamine lots commercially available, differences in animal strains, minor variations in rates of injection, or to seasonal changes in the sensitivity of the guinea pigs.

II. Influence of the Concentration and Duration of Pyribenzamine Aerosol Inhalation.

¹ Kallos, P., and Pagel, W., *Acta Med. Scandinav.*, 1937, **91**, 292.

² Schaumann, O., *Arch. f. Exp. Path. u. Pharmacol.*, 1940, **196**, 109.

³ von Issekutz, B., and Geuersich, P., *Arch. f. Exp. Path. u. Pharmacol.*, 1943, **202**, 201.

Inhalation of aerosols produced by vaporizing 0.5% and 1.0% PBZ solutions for various periods of time conferred definite protection against histamine to a few animals. A number of animals, however, did not show any significant beneficial response when treated with these relatively low concentrations.

Consistent results were obtained in almost all animals when the concentration of PBZ in the vaporizing fluid was increased to 2%. 34 animals were submitted to inhalations of 2% PBZ aerosol of varying duration. The shortest time of inhalation producing a definite protective effect against 2-3 lethal doses of histamine was between 1 and 2 minutes. With 2 minutes of inhalation, the animals tolerated intracardial injection of 2 mg histamine phosphate per kg, constituting approximately 2.5 lethal doses, without any symptoms of shock. With 3 minutes of inhalation they tolerated approximately 3.75 mg histamine phosphate (5 MLD), while inhalation for 5 to 10 minutes produced a resistance to 5-11 mg histamine phosphate (7 to 15 MLD).

The guinea pigs so protected did not go into shock and survived; however, all animals which were protected from typical histamine shock and had received histamine doses ranging from 5 to 11 mg per kg body weight presented very unusual signs of histamine intoxication. A few minutes after histamine injection the animals became motionless and then rapidly fell into deep narcosis with regular respiration, although very superficial and slow; there were also occasional athetotic movements of all legs. This interesting form of histamine intoxication is not normally observed, since death occurs from bronchoconstriction and asphyxia before narcosis could develop.

III. *Duration of Protection with 2% Pyribenzamine Aerosols.* Three series of 10, 16, and 10 animals were submitted to PBZ aerosol inhalation of 15, 30, and 60 minutes duration respectively. At varying intervals after completion of the PBZ treatment 2 animals of each series received an intracardial injection of 3 mg per kg histamine phosphate (4 MLD).

In the first series of experiments in which the 2% PBZ aerosol was inhaled for 15 minutes, the protection afforded against subsequent injection of 3 mg of histamine phos-

TABLE I.
Duration of Protection in Guinea Pigs Against 4 Lethal Doses of Histamine Phosphate After PBZ Aerosol Inhalation (2%) for 15, 30 and 60 Minutes.

Interval between end of PBZ inhalation and histamine injection	No. of animals protected		
	Duration of aerosol inhalation		
	15 min	30 min	60 min
0	2/2*		
15 min	2/2		
30 "	2/2	2/2	2/2
45 "	2/2	2/2	2/2
1 hr	2/2	2/2	1/2
2 "		2/2	2/2
3 "		1/2	2/2
3½ "		2/2	
4 "		0/4	
Controls, no PBZ		0/6	

* Number of animals protected and surviving over number of animals used.

phate (4 MLD) lasted for at least 1 hour (Table I). Inhalation of PBZ aerosol for 30 minutes gave protection lasting for 3½ hours, and inhalation for 60 minutes did not further increase the protection.

Anaphylaxis. 1. Influence of the duration of Pyribenzamine Aerosol Inhalation. Animals actively sensitized to horse serum or passively sensitized with anti-horse rabbit serum could be effectively protected by 2% PBZ aerosol inhalations. The shortest time of inhalation protecting animals against immediate anaphylactic shock was found to be 3 minutes. Animals challenged immediately after this time showed delayed symptoms of shock, but invariably died after 10 to 30 minutes. Complete protection against both shock and anaphylactic death was conferred after an inhalation period of at least 10 minutes.

Since the amounts of histamine or histamine-like substances liberated in the lungs during anaphylactic shock are not known, a direct comparison between the duration of PBZ aerosol inhalation necessary for protection against histamine poisoning and anaphylactic shock is not possible. It is interesting to note, however, that the results in anaphylaxis were somewhat less regular than those obtained in the histamine-shock series, since some animals died in shock even after being submitted to 2% PBZ aerosol inhalation of 30 minutes duration. Contrary to those animals protected against histamine, certain individuals,

TABLE II.

Duration of Protection in Guinea Pigs Against Anaphylactic Shock After PBZ (2%) Aerosol Inhalation for 15 and 30 Minutes.

Interval between end of PBZ inhalation and challenging serum inj.	Number of animals protected		
	Active anaphylaxis after PBZ inhalation of		Passive anaphylaxis after PBZ inhalation of
	15 min	30 min	30 min
15 min	1/2		3/3
30 "	3/4	2/3	3/3
45 "	0/3	2/3	2/3
60 "	2/3	1/2	3/3
2 hr		1/2	2/2
3 "		2/2	1/1
5 "			2/2
Controls, no PBZ	0/8		0/3

while protected against immediate anaphylactic shock by adequate treatment, died during the ensuing 24 hours.

II. *Duration of the Protection with 2% Pyribenzamine Aerosol Inhalation.* The duration of the protection to anaphylactic shock conferred to guinea pigs by PBZ aerosol inhalation was tested in the same manner as described in the experiments with histamine. Two groups of 12 sensitized animals each were submitted to an inhalation of 2% PBZ aerosol for 15 and 30 minutes respectively. It was found that inhalation of 15 minutes duration afforded protection up to 1 hour; while inhalation for 30 minutes gave protection for at least 3 hours. Protection was even greater and more consistent in 17 passively sensitized animals, since aerosol inhalation for 30 minutes was still effective after an interval of 5 hours (Table II).

III. *Influence of Aerosols Containing Trasentin or Procaine upon Histamine Intoxication.* PBZ and other antihistaminic substances display, in addition to the antihistaminic activity, powerful local anesthetic and variable antispasmodic activities. The possibility has been suggested that the activity of the antihistaminic substances, especially their effectiveness in anaphylaxis and allergy, are at least partially due to these secondary activities. Although it is known that local anesthetics and antispasmodics, such as atropine, are capable of protecting animals to a limited degree against histamine poisoning and anaphylactic shock, there is no proof that either of these secondary activities associated

with antihistaminics contribute to any significant degree to the antihistaminic and anti-anaphylactic activities of PBZ and similar substances. Nevertheless, it seemed interesting to substitute for aerosols of PBZ, aerosols of substances with pronounced antispasmodic and local anesthetic activity and to investigate their influence upon histamine intoxication. We found that neither 2% procaine nor 2% Trasentin aerosol inhalation had any influence upon histamine intoxication using 2 to 3 lethal doses of histamine; convulsions and death occurred at the same intervals as in control animals not submitted to these aerosols.

IV. *Local Action of Pyribenzamine Inhalation upon the Lungs.* As it is known that various antihistaminic substances are irritating when injected subcutaneously, it was therefore necessary to control the effect of PBZ aerosol inhalation upon the cells of the lung system. Guinea pigs were subjected to inhalation of 2% PBZ for up to 2 hours and then observed for several days. The treatment was well tolerated and did not produce any clinical signs of irritation either during treatment, or subsequently. Three other animals, after receiving a 1-hour aerosol treatment, were killed 24 hours later and the lungs histologically examined. They were found to be normal.

Discussion. These experiments indicate that it is possible to protect guinea pigs against 15 lethal doses of histamine, as well as against anaphylactic shock when 2% PBZ is administered in the form of an aerosol. Under the conditions of our experiments, the animals were protected after having inhaled, by calcu-

lation, 0.3 to 0.5 mg PBZ, which may have been absorbed, in whole or in part, by the lungs. This dose, as we have found from all our previous experiments, is ineffective when either injected subcutaneously or given orally. These results constitute, in our opinion, further evidence supporting the hypothesis that PBZ specifically counteracts histamine not by central action, but by a purely local influence at the very site of the peripheral histamine action.^{2,3,7,8}

The evidence that the point of attack by PBZ is the peripheral receptor may explain why the protection against histamine intoxication or anaphylactic shock, persists for a surprisingly long period of time in spite of the exceedingly small amounts of PBZ which reach the lung tissue during the brief aerosol treatment. Indeed, the protective effect lasts almost as long as that protection which is conferred when PBZ is given subcutaneously or by mouth in considerably higher dosages.

It may be concluded from our results that PBZ is either selectively fixed within the receptor cells of the lungs and does not rapidly enter the blood stream as do most other substances introduced therapeutically into the lungs, or that the minute amounts of antihistaminic substances, by direct contact with the cells susceptible to histamine, render the receptive organs, by some functional mechanisms, refractive for long periods of time to subsequent attacks by histamine.

The introduction of antihistaminic substances into the lungs and the selective protection of this organ against histamine in guinea pigs have enabled us to detect signs of histamine intoxication previously not observed. Under normal conditions, the picture of histamine intoxications in guinea pigs consists almost entirely of the constriction of smooth muscles and its immediate consequences, and before any other signs can develop, the animal dies of asphyxia. On the other hand, smaller doses which do not produce this type of death are apparently too small to produce other

symptoms. However, when the animals are protected against death by asphyxia, as in the present experiments, histamine in significantly high dosage can be introduced and unusual symptoms are observed, as described in these experiments; namely, a pronounced narcotic effect which lasts from 30 minutes to 1 hour. At the end of this period the animals slowly recover, as from deep narcosis.

It may be more than a fortuitous occurrence that in certain patients the various antihistaminic substances exert a definite depression and an almost narcotic action. This symptom may be due to the local anesthetic power of these substances. It also could be explained in the light of the foregoing observations; namely, that the competitive action of PBZ with histamine, in some instances, may lead to a duplication of certain histamine effects; while in others, to a suppression of other typical histamine activities.

PBZ aerosol inhalation not only confers a powerful protection against histamine intoxication in guinea pigs, but also protects against anaphylaxis. The doses necessary to prevent anaphylactic shock are approximately the same as those which are active against histamine poisoning, and in both cases the duration of protection is essentially the same. The fact that exceedingly small doses of PBZ introduced directly into the lungs are equally effective in both histamine poisoning and anaphylaxis adds another argument in favor of the theory that anaphylactic death and histamine death in guinea pigs are produced by the same poison, or at least by substances closely related.

Conclusions. 1. PBZ inhaled in the form of an aerosol and in minute amounts, protects guinea pigs against histamine intoxication and anaphylactic shock. This protection is relatively long-lasting. 2. The selective protection which PBZ aerosols confer to the lung tissue permits the observation in guinea pigs of hitherto unrecorded histamine effects. 3. The high activity of PBZ aerosol inhalation and the apparent lack of irritation suggest the possible therapeutic use of PBZ as an aerosol, since it may be predicted that this method of administration produces a lasting therapeutic effect with doses too small to produce undesirable side reactions.

⁷ Wells, J. A., Morris, H. D., Bull, H. B., and Dragstedt, C. A., *J. Pharm. Exp. Ther.*, 1945, **85**, 122.

⁸ Yonkman, F. F., Chess, D., Mathieson, D., Hansen, N., *J. Pharm. Exp. Ther.*, 1946, **87**, 256.

In vitro Observations of the Mode of Action of Streptomycin.*

ELIAS STRAUSS. (Introduced by S. Edward Sulkin.)

From the Department of Bacteriology and Immunology and the Department of Medicine, Southwestern Medical College, Dallas.

Despite wide clinical interest in the use of streptomycin, the mode of action of this substance on bacteria is as yet poorly understood. The present report deals with 3 aspects of the action of streptomycin on bacteria *in vitro*: (1) morphological effects of streptomycin on growing cultures of various organisms; (2) the bactericidal action of streptomycin on resting bacteria; and (3) the effect on the rate of growth of bacteria of subinhibitory concentrations of streptomycin.

Effect of Streptomycin on Morphological Appearance of Bacteria. Little attention has been directed toward possible alterations in bacterial morphology as a result of growth in the presence of streptomycin. Welch, Price, and Randall¹ observed huge and bizarre forms of *E. typhosa* in cultures exposed to streptomycin. However, Miller and Bohnhoff² found no morphological changes in meningococci or gonococci grown in the presence of streptomycin.

In the present study, a single stock laboratory strain of each of the following organisms was employed: *Aerobacter aerogenes*, *Escherichia coli*, *Proteus ammoniac*, *Pseudomonas aeruginosa*, *Shigella sonnei*, *Eberthella typhosa*, *Salmonella typhimurium*, *Vibrio camma*, *Micrococcus tetragenus*, *Staphylococcus aureus*, *Bacillus anthracis*, *Bacillus mycoides*, *Bacillus subtilis*, and *Bacillus megatherium*. Preliminary tests were performed in heart infusion broth by the serial dilution technic to determine the range of sensitivity of each organism to streptomycin. The degree of bacteriostasis was estimated by the presence

or absence of turbidity after 24 hours' incubation. In the final tests, 10⁻² dilutions of overnight growths of organisms in broth were incubated at 37°C in heart infusion broth in the presence of various amounts of streptomycin, which, on the basis of the preliminary study, were expected to be slightly, moderately, or strongly bacteriostatic. Replicate tubes were prepared for each organism with the several streptomycin concentrations, as well as control tubes. At 0, 3 and 5 to 6 hours' incubation, the tubes were centrifuged for 20 minutes and Gram-stained smears of the bacterial sediment prepared. In each instance, a streptomycin-free culture was smeared and stained on the same slide as the streptomycin-containing culture. The stained smears were examined independently by 2 observers, one of whom was unaware of the purpose or technic of the study.

The effect of streptomycin on the size, shape and staining qualities of bacteria was variable, being marked with some Gram-negative bacilli, slight with Gram-positive cocci, and absent with Gram-positive bacilli. The most extreme alterations in appearance were found with the strain of *Shigella sonnei*. Cells of this organism, when exposed to streptomycin, became elongated (up to 3 or 4 times as long as the control cells), appeared swollen, and stained irregularly. (Fig. 1). These alterations were more pronounced with a slightly bacteriostatic amount of streptomycin (1 γ per ml) than with a higher, more strongly bacteriostatic concentration (5 γ per ml). Cells of changed appearance were somewhat more frequent after 6 hours' incubation than after 3 hours.

Cells of the strain of *S. typhimurium*, when exposed to streptomycin, became swollen and elongated (up to 2 or 3 times the normal length), and assumed coccoid forms. These changes were equally prominent after 3 and 6 hours' incubation. The proportion of altered cells to the total was greater with

* This investigation was supported by grants from the Lederle Laboratories Division, American Cyanamid Company, and from the United States Public Health Service.

¹ Welch, H., Price, C. W., and Randall, W. A., *J. Am. Pharm. Assn.*, 1946, **35**, 155.

² Miller, C. P., and Bohnhoff, M., *J. Am. Med. Assn.*, 1946, **130**, 485.

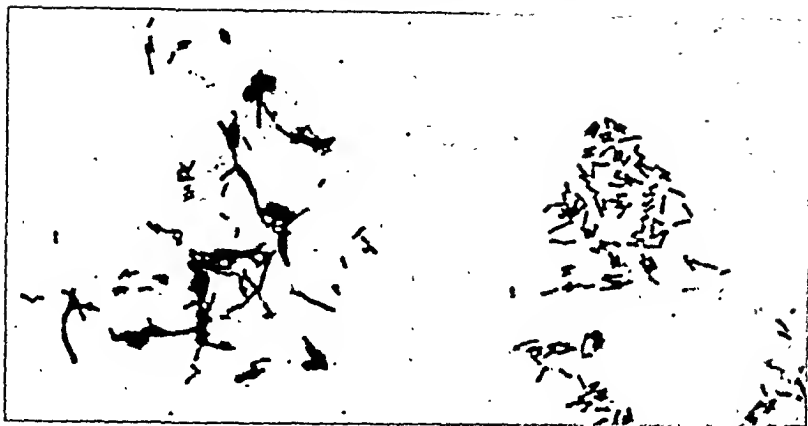


FIG. 1.

Shigella sonnei ($\times 675$), Gram-stained smears after 6 hours' incubation at 37°C in heart infusion broth. Left: In the presence of streptomycin, 1.0γ per ml. Right: Control, without streptomycin.

slightly bacteriostatic concentrations of streptomycin (3 and 6γ per ml) than with a strongly bacteriostatic concentration (12γ per ml).

Definite, but lesser, degrees of alteration in cellular appearance were observed with the strains of *Acrobacter aerogenes* and *Proteus ammoniac*. In both instances incubation with streptomycin for either 3 or 6 hours resulted in a greater proportion of moderately elongated and swollen cells than were observed in control cultures incubated in the same manner without streptomycin. Again the changes were somewhat more frequent in slightly bacteriostatic amounts of streptomycin (1γ and 3γ per ml) than in more strongly bacteriostatic concentrations (6γ and 12γ per ml).

The other Gram-negative organisms exhibited little or no alteration in appearance. *Vibrio comma* appeared somewhat more pleomorphic in the presence of streptomycin than in its absence. No consistent changes were observed in the appearance of *Escherichia coli*, *Eberthella typhosa*, and *Pseudomonas aeruginosa*.

A few swollen and deeply-stained cells of *Staphylococcus aureus* and *Micrococcus tetragenus* were found in cultures containing streptomycin but such cells were also seen with nearly the same frequency in control cultures. The Gram-positive bacilli studied exhibited no consistent alterations in shape, size, or staining as a result of incubation with

EFFECT OF STREPTOMYCIN ON SUSCEPTIBLE AND RESISTANT STRAINS OF *E. COLI* IN RINGER'S SOLUTION

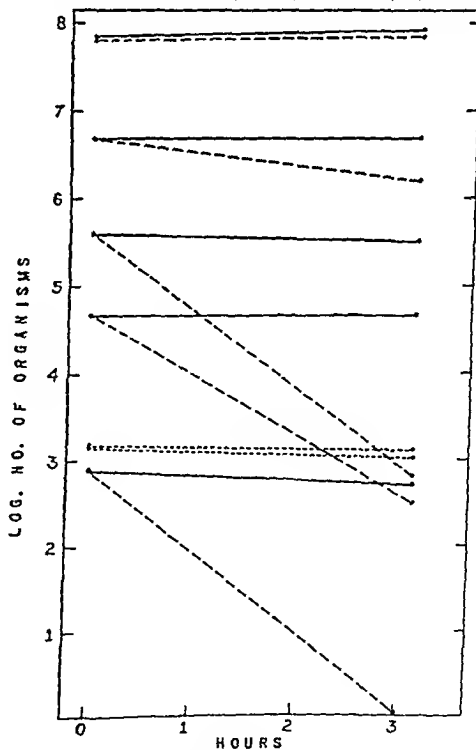


FIG. 2.

Bactericidal action of streptomycin on sensitive and resistant strains of *E. coli* suspended in Ringer's solution at 37°C . Solid lines: Sensitive strain, without streptomycin. Broken lines: Sensitive strain, with streptomycin, 25γ per ml. Dotted lines: Resistant strain, without streptomycin, and with streptomycin 25γ per ml.

streptomycin.

Bactericidal Action of Streptomycin on Resting Bacteria. Hamre, Rake, and Donovick³ reported that streptomycin was bactericidal for *K. pneumoniae* in Ringer's solution. The effect was related to the concentration of streptomycin employed. A greater concentration was required for bactericidal action when the bacterial cells were in the resting state than when they were actively growing. Resting cells surviving 3 or 5 hours' incubation with streptomycin subsequently were not found more resistant than controls. It was suggested, therefore, that the mode of action of streptomycin on washed, resting cells is different from its action on multiplying organisms. Klein and Kimmelman⁴ also noted that streptomycin was bactericidal for washed suspensions of *Shigellae* and that the survivors did not show increased resistance. The present studies were undertaken to describe more fully the bactericidal action of streptomycin on resting bacteria and to ascertain whether or not its mode of action under these circumstances differed from its action on multiplying organisms.

A stock laboratory strain of *E. coli* was used. The sedimented organisms from a 6-hour growth in heart-infusion broth were washed twice in Ringer's solution (pH 7.0), and resuspended in Ringer's solution after dilution to the desired bacterial concentrations. Streptomycin in Ringer's solution was added to some of the tubes and the volumes of both control and drug-containing tubes were adjusted. Incubation was carried out in a water bath at 37°C for 3 hours. Explants were made at 0 and 3 hours in a simple synthetic medium⁵ to which were added agar, glucose (1%), Bacto tryptose (1%) and sodium thioglycolate (0.05%). Preliminary tests indicated that in this medium concentrations of streptomycin up to 5 γ per ml had no inhibitory effect on the number of developing colonies as compared with controls. In heart-infusion broth as-

says, the strain was inhibited by 1.6 γ per ml or less of streptomycin.

The results of one set of experiments are shown in Fig. 2. The bactericidal action of streptomycin was related, in general, to the concentration of organisms. Bacterial suspensions of 1000 per ml or less were sterilized in 3 hours by 25 γ per ml of streptomycin; suspensions of 10 million or more cells per ml were not affected by this concentration of streptomycin in the same time interval. Intermediate numbers of organisms were affected proportionately. The activity of 50 γ per ml of streptomycin was greater and that of 10 γ per ml was correspondingly less than the effects indicated with 25 γ per ml. The amount of streptomycin required to sterilize a resting suspension of *E. coli* within 3 hours was approximately 25 times as great as the bactericidal concentration for a similar number of organisms incubated in heart-infusion broth.

An attempt was made to increase the resistance of *E. coli* by repeated exposure of resting organisms to sublethal concentrations of streptomycin. After growth in heart-infusion broth the organisms were washed twice, resuspended in Ringer's solution containing 10 γ per ml of streptomycin, and incubated for 3 hours at 37°C. A loopful of the bacterial suspension was then streaked on an agar plate. After incubation the growth from the agar was again washed and resuspended for 3 hours in Ringer's solution containing 10 γ per ml of streptomycin. This procedure was carried out through 12 passages. A control strain was treated in an identical manner except for the omission of streptomycin. At the completion of the series, both strains were tested for resistance to streptomycin. Neither exhibited any increased resistance to the bactericidal action of streptomycin, as compared with unexposed control strains, when tested in the resting state or when incubated in heart-infusion broth. These results, which are in agreement with the reports of others, suggest a difference in the mode of action of streptomycin on resting and on multiplying organisms.

To further elucidate the mechanism of the mode of action of streptomycin, observations

³ Hamre, D., Rake, G., and Donovick, R., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 25.

⁴ Klein, M., and Kimmelman, L. J., *J. Bact.*, 1946, **52**, 471.

⁵ Sahyun, M., Beard, P., Schultz, E. W., Snow, J., and Cross, E., *J. Infect. Dis.*, 1936, **58**, 28.

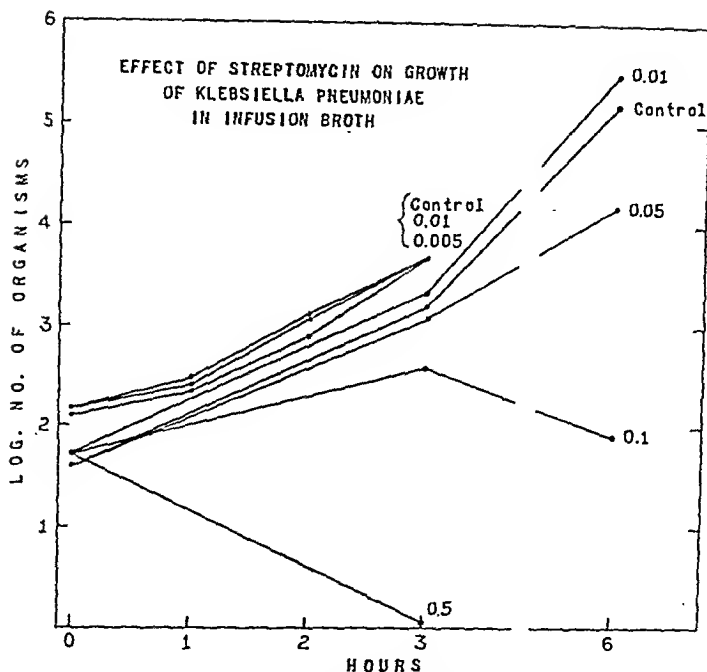


FIG. 3.

Rate of growth of *K. pneumoniae* in heart-infusion broth at 37°C in presence of bactericidal, bacteriostatic, and subinhibitory concentrations of streptomycin. Numbers indicate γ per ml of streptomycin.

were made of the behavior of a strain of bacteria made resistant to streptomycin and subsequently exposed in the resting state to the antibiotic. Accordingly, the stock strain of *E. coli* was made resistant by repeated passage in increasing concentrations of streptomycin in tryptose-phosphate broth. After 12 daily passages, the organisms grew freely in 150 γ per ml of streptomycin. The resistant organisms were then suspended in Ringer's solution and exposed to streptomycin by the procedure already described. A representative result is shown in Fig. 2. This strain was completely resistant to 25 γ per ml of streptomycin—an amount of streptomycin which was bactericidal for a similar number of nonresistant organisms. The mechanism of bacterial resistance to chemotherapeutic agents is presumed to be intimately related to the mechanism of the antibacterial action of such drugs. These results, therefore, suggest that the mode of action of streptomycin on nonmultiplying organisms is essentially similar to its action on

multiplying organisms.

Effect of Subinhibitory Concentrations of Streptomycin on Rate of Growth. On the basis of *in vivo* studies in mice, the suggestion was advanced that subinhibitory concentrations of streptomycin might have a stimulatory effect on the rate of growth of *E. typhosa*.¹ This possibility was investigated *in vitro* using a stock strain of *K. pneumoniae*. The organisms were grown overnight in heart-infusion broth, suitably diluted, and inoculated in broth containing various amounts of streptomycin. Explants in tryptose-phosphate agar were made at intervals after incubation at 37°C, and the subsequent growth of colonies counted. The results of 2 experiments are shown in Fig. 3. With this strain of bacteria, 0.5 γ per ml of streptomycin was bactericidal in 3 hours, and 0.1 and 0.05 γ per ml were moderately or slightly bacteriostatic. Lesser concentrations of streptomycin did not appreciably modify the rate of growth of the organisms as compared with the drug-free controls. Colony counts

on explants made at hourly intervals during the early phases of growth likewise yielded no evidence of an initial stimulus to the rate of growth from the presence of subinhibitory concentrations of streptomycin.

Comment. The morphological changes in bacteria resulting from streptomycin appear to be less marked than those which occur with exposure to penicillin.⁶⁻⁹ Like the latter, however, they occur with concentrations of streptomycin which are only slightly bacteriostatic. Alterations in morphology are not regularly associated with bacteriostasis. The growth of all of the organisms studied was inhibited by streptomycin, in concentrations varying between 1 and 50 γ per ml, but only a few strains exhibited abnormalities in size and shape.

Because of its pronounced effect on bacterial morphology, as well as its failure to sterilize nonmultiplying cultures, penicillin is presumed to interfere directly with bacterial fission. In the case of streptomycin it is suggested that the observed morphological changes may be secondary effects resulting from disturbances of intermediary metabolism rather than primary effects on fission. Such an hypothesis is also consistent with the observed bactericidal action of streptomycin on resting, nonmultiplying cells.

The failure of resting cells to develop re-

sistance to streptomycin and the greatly increased concentrations of antibiotic required to effect sterilization in the resting state, as contrasted with the multiplying state, suggest that streptomycin may act on a different locus or by a different mechanism under the 2 conditions of study. However, cells made resistant to streptomycin under conditions of growth are also resistant in the resting state. This observation appears to support the view that the mechanism of action of streptomycin on resting and on multiplying cells is fundamentally the same, the observed differences in behavior being accounted for on the basis of differences in the rate of bacterial metabolism.

Summary. 1. Streptomycin in bacteriostatic concentrations produced morphological changes in some Gram-negative bacilli but not in others. Gram-positive cocci were only slightly affected, and Gram-positive bacilli were not affected. 2. Streptomycin was bactericidal for *E. coli* in the resting state, the effect being related both to the size of the inoculum and the concentration of the antibiotic. 3. Strains of *E. coli* resistant to streptomycin under conditions permitting growth were also resistant in the resting state, but exposure of susceptible strains to streptomycin in the resting state did not result in increased resistance. 4. No stimulation of the rate of growth of *K. pneumoniae* was observed *in vitro* by subinhibitory concentrations of streptomycin.

Grateful acknowledgment is made to Elizabeth Watson for assistance with the morphological studies.

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Similarities in Electron Micrographs of Purified Lansing and SK Polio-myelitis Virus.

HUBERT S. LORING.

From the Department of Chemistry and the School of Medicine, Stanford University, California.

This note is written to correct the impression which was erroneously given¹ that purified

SK poliomyelitis virus consists of asymmetric particles of the type described by

¹ Loring, H. S., Marton, L., and Schwerdt, C. E.

PROC. SOC. EXP. BIOL. AND MED., 1946, 62, 281.

Gard² for a strain of Theiler's mouse virus. While this conclusion was suggested by one of Bourdillon's³ experiments in which an older dialyzed, purified preparation showed double refraction of flow, the results published by Jungeblut and Bourdillon⁴ show a remarkable agreement between purified SK and our purified Lansing preparations. The purified SK samples in contrast to those from

unpurified tissue cultures showed the presence of "spherical or elliptic bodies" having a diameter between 25 and 30 m μ . It is noteworthy that particles of the same relative size and shape have been obtained, therefore, from 2 different strains of poliomyelitis virus, both of which were isolated originally from infectious human material. As it might be expected that different human strains would have similar physical properties, the results from the 2 laboratories confirm the fact that human poliomyelitis virus as cultivated in mice and in cotton rats consists of relatively spherical particles of about 25 m μ diameter.

² Gard, S., *Acta Med. Scand.*, Suppl., 1943, 143, 173p.

³ Bourdillon, J., *Arch. Biochem.*, 1943, 3, 285.

⁴ Jungeblut, C. W., and Bourdillon, J., *J. Am. Med. Assn.*, 1943, 123, 399.

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Further Studies of Factor R.*

L. W. CHARKEY,[†] LOUISE J. DANIEL, FLORENCE A. FARMER, L. C. NORRIS, AND G. F. HEUSER.

From the Agricultural Experiment Station and the School of Nutrition, Cornell University, Ithaca, N.Y.

Bauernfeind, Schumacher, Hodson, Norris, and Heuser¹ announced the existence of a new factor required for growth and reproduction in the domestic fowl. Schumacher, Heuser, and Norris² presented evidence that the factor consisted of 2 components; factor S precipitated from an acid solution by alcohol, and factor R precipitated by alcohol from neutral solution.

While these studies on factors R and S were in progress, evidence of the existence of a factor required for the growth of *L.*

casei and *S. jaccalis* was presented by various laboratories. This factor is now generally referred to as folic acid. Briggs, Luckey, Elvehjem, and Hart³ reported that chicks grew normally on a diet containing no more than 8 μ g of folic acid per 100 g of diet. Hill, Norris, and Heuser⁴ concluded that factor R was not identical with folic acid, and that if folic acid was required by the chick, the amount needed was less than 15 μ g per 100 g of diet.

Binkley and associates⁵ reported the presence of vitamin B_c (folic acid) conjugate in yeast. This substance was highly active for the chick, but only slightly active for *S.*

* This work was supported in part by the establishment of grants at Cornell University by the Cerophyl Laboratories, Inc., Kansas City, Mo., The Nutrition Foundation, Inc., New York, N.Y., and the Wyeth Institute of Applied Biochemistry, Philadelphia, Pa.

[†] Present address, Department of Chemistry, Colorado A. and M. College, Fort Collins, Colo.

¹ Bauernfeind, J. C., Schumacher, A. E., Hodson, A. Z., Norris, L. C., and Heuser, G. F., *Proc. Soc. Exp. Biol. and Med.*, 1938, 39, 108.

² Schumacher, A. E., Heuser, G. F., and Norris, L. C., *J. Biol. Chem.*, 1940, 135, 313.

³ Briggs, G. M., Jr., Luckey, T. D., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1945, 158, 303.

⁴ Hill, F. W., Norris, L. C., and Heuser, G. F., *J. Nutrition*, 1944, 28, 175.

⁵ Binkley, S. B., Bird, O. D., Bloom, E. S., Brown, R. A., Calkins, D. G., Campbell, C. J., Emmett, A. D., and Piffner, J. J., *Science*, 1944, 100, 36.

faecalis and *L. casei*. Concentrates of this substance, however, became active for the microorganisms after enzymatic digestion.

In view of the results of Binkley and associates,⁵ further work on factor R was undertaken in this laboratory to determine if its activity for chicks could be accounted for in terms of folic acid, as measured by microorganisms after enzymatic conversion of the conjugate to folic acid.

While the study reported in this paper was in progress, Pfiffner and associates⁶ reported the isolation of vitamin B₉ conjugate in crystalline form from yeast. The vitamin B₉ present in the conjugate was found to be available to the chick, and became available to the microorganism after digestion with hog kidney conjugase.

Experimental. The experimental procedure consisted of treatment of factor R with folic acid liberating enzymes, determination by microorganisms of the folic acid in the treated preparations, and comparison with the amount of folic acid present as evidenced by growth and hemoglobin formation in the chick.

Microbiological Work. Folic acid assays were made by a modification of the procedure of Luckey, Briggs, and Elvehjem⁷ using *S. faecalis*. The standard of comparison was either synthetic folic acid of Angier and associates⁸ or a folic acid concentrate supplied by Dr. R. J. Williams. One μ g of synthetic folic acid was found to be equivalent

to 11.5 μ g of Williams' folic acid. Values, obtained when Williams' folic acid was used as the standard, were converted to synthetic folic acid by means of this factor.

The amount of free folic acid in factor R was determined following either incubation of the sample with takadiastase and papain or steaming in phosphate buffer at pH 7 for 15 minutes. Both methods of extraction gave comparable values. The results showed that there was not sufficient folic acid present as such to account for the growth response of the chick to factor R. Therefore, other enzymatic digestions were undertaken to determine the presence of potential or conjugated folic acid that was microbiologically inactive, but available to the chick.

In preliminary work the most effective procedure found was one involving a 24-hour predigestion at 37°C with malt diastase, followed by a 24-hour incubation at 37°C with fresh rat liver suspension. The entire procedure was carried out in citrate-phosphate buffer at pH 4.5. This treatment was applied to factor R and to a Superfiltrol eluate of factor R, and consistently gave an approximately 10-fold increase in folic acid content. Chick liver, in general, was less effective than rat liver. Increases of this order of magnitude still, however, did not adequately account for all the biological activity of factor R. Hence, resort was made to the use of dried kidney suspension, as described by Bird and associates.⁹ This procedure resulted in an approximately 15-fold increase in folic acid content.

Chick Experiments. Two experiments were conducted with day-old White Leghorn chicks. Not less than 15 chicks were started in each lot. They were housed in batteries of individually heated, well lighted pens with raised wire mesh bottoms.

The basal diet used was purified diet 653 described by Hill, Norris and Heuser.⁴ Factor S, prepared according to the method of Schumacher, Heuser, and Norris,² was supplied at a level equivalent to 5% of dried brewers' yeast, and 100 μ g of 2-methyl-3-

⁶ Pfiffner, J. J., Calkins, D. G., O'Dell, B. L., Bloom, E. S., Brown, R. A., Campbell, C. J., and Bird, O. D., *Science*, 1945, **102**, 228.

⁷ Luckey, T. D., Briggs, G. M., Jr., and Elvehjem, C. A., *J. Biol. Chem.*, 1944, **152**, 157.

⁸ Angier, R. B., Boothe, J. H., Hutchings, B. L., Mowat, J. H., Semb, J., Stokstad, E. L. R., Subbarow, Y., Waller, C. W., Cosulich, D. B., Fahrenbach, M. J., Hultquist, M. E., Kuh, E., Northey, E. H., Seeger, D. R., Sickels, J. P., and Smith, J. M., Jr., *Science*, 1945, **102**, 227.

⁴ We are indebted to Lederle Laboratories, Pearl River, N.Y., for synthetic folic acid, to Dr. R. J. Williams, University of Texas, Austin, Tex., for folic acid concentrate, to Merek & Co., Inc., Rahway, N.J., for β -pyracin, and to Anheuser-Busch, Inc., St. Louis, Mo., for strain S dried brewers' yeast.

⁹ Bird, O. D., Bressler, B., Brown, R. A., Campbell, C. J., and Emmett, A. D., *J. Biol. Chem.*, 1945, **159**, 631.

hydroxy-4-carboxy-5-hydroxymethylpyridine (β -pyracin) were added per 100 g of diet.

The chicks were weighed individually at weekly intervals. Hemoglobin determinations were made at the 3rd, 4th, and 6th weeks in Experiment 1 by the colorimetric method of Sahli, using 0.1 N HCl to develop the acid hematin color, and in Experiment 2 by the oxyhemoglobin method developed in this laboratory by Robertson and Fiala,¹⁰ using 0.1% Na_2CO_3 and measuring the color produced with a Coleman spectrophotometer.

Experiment 1. The *L. casei* factor of Hutchings and associates,¹¹ was used as a standard. The sample used was approximately 80% pure and was added at levels of 50, 100 and 150 μg per 100 g of diet. Factor R was included at levels equivalent to 5, 10, and 15% of original yeast. The results of the experiment indicated that 50 to 100 μg of *L. casei* factor were needed for optimum growth response in the chick. Using the same experimental conditions, Robertson, Daniel, Farmer, Norris, and Heuser¹² have reported that 45 to 55 μg of synthetic folic acid per 100 g of diet were required for optimum growth. Normal growth was obtained with factor R preparations that supplied approximately 25 μg of folic acid per 100 g of diet, as determined by *S. faecalis* assay after rat liver incubation.

Experiment 2. When synthetic folic acid became available, an experiment was conducted using 10, 20, 30, and 40 μg of folic acid per 100 g of diet. Factor R was included at levels equivalent to 3, 6, and 12% of original yeast. One group of chicks was supplied the various supplements alone, and a second group the supplements plus 2% succinylsulfathiazole. When the sulfonamide was added, *p*-aminobenzoic acid was omitted from the diet. The results of Experiment 2 are presented in Table I.

The amount of folic acid found in factor R

by this chick experiment was approximately 3 μg per g of original yeast. This was obtained by comparing both the growth and hemoglobin responses at the various levels of factor R to the corresponding responses caused by synthetic folic acid. Comparing the biological and microbiological responses to factor R demonstrated that the microorganism was accounting for all of the chick activity after the sample had been incubated with hog kidney, but not when incubated with chick or rat liver.

No significant differences in the chick responses to factor R between the nonsulfonamide and sulfonamide diets were obtained. This indicated that potential folic acid in factor R was not liberated by intestinal bacteria and that under the conditions of the experiment no folic acid was synthesized by the intestinal flora. It appeared, therefore, that either the chick is able to use the folic acid of factor R directly, or metabolic processes make it available.

Discussion. Several explanations for the differences in microbiological results obtained by incubating factor R with chick or rat liver and hog kidney are possible. More than one conjugate of folic acid may be present in factor R. If this is the case, the hog kidney enzyme(s) is able to break down both conjugates, whereas rat and chick liver enzymes can split only one.

The presence of more than one conjugate of folic acid in factor R is supported by the recent findings of Ratner, Blanchard, and Green.¹³ These investigators isolated a peptide of *p*-aminobenzoic acid from yeast having 10 or 11 glutamic acid residues. In one of the steps in the isolation of this peptide trichloroacetic acid was used. This treatment may have split the peptide from the pterin nucleus of the folic acid conjugate, since Angier and associates¹⁴ brought about the

¹⁰ Robertson, E. I., and Fiala, G. F., unpublished data, Cornell University, Ithaca, New York.

¹¹ Hutchings, B. L., Stokstad, E. L. R., Bohonos, N., and Slobodkin, N. H., *Science*, 1944, **99**, 371.

¹² Robertson, E. I., Daniel, L. J., Farmer, F. A., Norris, L. C., and Heuser, G. F., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 97.

¹³ Ratner, S., Blanchard, M., and Green, D. E., *J. Biol. Chem.*, 1946, **164**, 691.

¹⁴ Angier, R. B., Boothe, J. H., Hutchings, B. L., Mowat, J. H., Semb, J., Stokstad, E. L. R., SubbaRow, Y., Waller, C. W., Cosulich, D. B., Fahrenbach, M. J., Hultquist, M. E., Kuh, E., Northey, E. H., Seeger, D. R., Sickels, J. P., and Smith, J. M., Jr., *Science*, 1946, **103**, 667.

TABLE I.
Effect of Factor R on Growth and Hemoglobin Formation in the Chick.

Effect of Factor R on Growth and Hemoglobin Formation in the Chick.									
Supplements	Weight		Hemoglobin		Folic acid added by supplement				
	4 wk		6 wk		Unincubated	Hog kidney	Rat liver	Chick liver	
Without succinylsulfathiazole.									
None*	153 (11)†	202 (5)	3.9	2.4	—	—	—	—	
10 µg folic acid‡	225 (15)	345 (12)	5.1	6.9	10	—	—	—	
20 " "	249 (15)	412 (15)	8.0	9.5	20	—	—	—	
30 " "	288 (15)	476 (15)	8.3	9.2	30	—	—	—	
40 " "	274 (14)	461 (14)	8.7	9.3	40	—	—	—	
3% factor R	174 (12)	266 (9)	3.7	5.1	0.6	9.9	7.5	6.0	
6 " "	241 (12)	445 (11)	7.8	9.8	1.2	19.8	15.0	12.0	
12 " "	298 (12)	492 (12)	8.4	9.4	2.4	39.6	30.0	24.0	
2% succinylsulfathiazole added.									
None	153 (8)	173 (2)	3.2	1.6	—	—	—	—	
10 µg folic acid	206 (12)	310 (11)	4.8	0.6	10	—	—	—	
20 " "	248 (14)	431 (14)	7.3	9.1	20	—	—	—	
30 " "	282 (14)	469 (14)	8.2	9.5	30	—	—	—	
40 " "	281 (14)	470 (14)	8.5	9.5	40	—	—	—	
3% factor R	186 (12)	230 (8)	4.4	3.5	0.6	9.9	7.5	6.0	
6 " "	239 (15)	413 (11)	6.2	9.1	1.2	19.8	15.0	12.0	
12 " "	301 (15)	492 (15)	8.0	9.2	2.4	39.6	30.0	24.0	

* Basal diet contained 15 µg of folic acid per 100 g as determined by *S. faecalis* assay following chick liver incubation.

† Numbers in parentheses indicate number of surviving chicks of the original 15.

‡ Synthetic folic acid.

cleavage of the fermentation *L. casei* factor giving a pteridine fraction and an aromatic amine by using sulfurous acid, which is a milder treatment than that with trichloroacetic acid.

The vitamin B₆ conjugate of Pfiffner and associates,¹⁵ which is also obtained from yeast, has been shown to contain 7 molecules of glutamic acid. This evidence indicates that there may be at least 2 different conjugates in yeast. If there are 2 conjugates of folic acid present in factor R, it may be that there are 2 enzymes needed to split them, both of which are present in hog kidney and only one in rat and chick liver.

All conditions necessary for the optimum efficiency of the rat and chick liver enzyme systems may not have been met, so that complete liberation of folic acid from the conjugate was not obtained. Every effort was made to determine the optimum conditions for the enzyme systems involved. The proper concentrations of the enzymes and substrate were determined, as well as the optimum pH and temperature for the incubation.

Hog kidney may contain an enzyme system that can synthesize folic acid from simple precursors, whereas chick and rat liver may be devoid of such an enzyme. Many

¹⁵ Pfiffner, J. J., Calkins, D. G., Bloom, E. S., and O'Dell, B. L., *J. Am. Chem. Soc.*, 1946, **68**, 1392.

bacteria, yeasts, and molds are known to possess enzyme systems that are able to take the pyrimidine and thiazole fractions of thiamine and combine them into the vitamin.

Summary. The growth and hemoglobin responses obtained in chicks receiving a purified diet supplemented with factor R were found not to be due to the preformed folic acid present in factor R.

By means of chick liver, rat liver, and hog kidney incubation procedures the folic acid content of factor R was greatly increased. This indicated the presence of potential folic acid which did not stimulate the growth of *S. faecalis*, but which was available to the chick. The potential folic acid liberated by hog kidney enzymes was found to account for all of the responses obtained in the chick, whereas chick and rat liver enzymes were not as effective in liberating folic acid.

The most probable explanation for the discrepancy in the folic acid content of factor R obtained following hog kidney and rat or chick liver incubations is that factor R is a mixture of conjugates of folic acid rather than one conjugate.

The response to factor R was not affected by the presence of succinylsulfathiazole in the diet. Conjugated folic acid appeared, therefore, to be available as such to the chick, or was made available by metabolic processes.

15714

Nerve Conduction After Inactivation of Choline Esterase.*

L. L. BOYARSKY, JULIAN M. TOBIAS, AND R. W. GERARD.

From the Department of Physiology, The University of Chicago.

Gilman reported in February, 1946¹ that di-isopropyl fluorophosphate (DFP) could block conduction in a stretch of frog nerve dipped in it; but that conduction returned on lifting out the nerve. Despite this "re-

versibility" of the block, the choline esterase (Ch.E.) was left inactivated. Objections were raised to the conditions of these experiments (atypical action potentials, manner of obtaining reversal, low frequency of stimula-

* The present investigation was aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

¹ *The Physics and Chemistry of Nerve Conduction*, New York Academy of Sciences Symposium Monograph, in press.

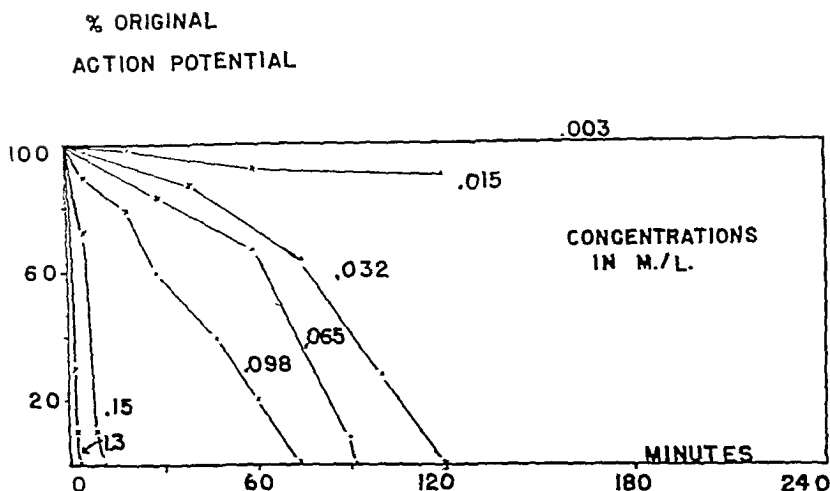


Fig. 1.

tion, small CO_2 values in the manometric determination of Ch.E., inactivation of Ch.E. at the time of grinding of the nerve, etc.).

A few weeks later, at the Federation symposium, succeeding papers by Crescitelli, Gilman, *et al.*^{2,3} and by Rothberg, Nachmansohn, *et al.*^{4,5} were the basis for an extensive further discussion. The former reaffirmed

their findings, with added details; the latter reported that DFP depressed action and Ch.E. in parallel, and that both recovered in parallel on removing the drug, in squid nerve. The attention of other workers was invited to this experiment which, with other evidence (Gerard¹), seemed potentially conclusive as to the role of A.Ch. in nerve conduction.

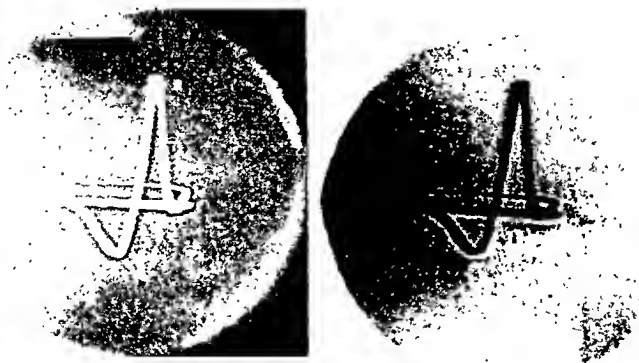


Fig. 2.

Left trace initial; right trace after 5 hours in 3 mM DFP. Read R to L. Conduction distance to first electrode 10 mm; to second one 30 mm. No crush, second electrode near cut end.

² Crescitelli, F., Koelle, G. B., and Gilman, A. F., *Fed. Proc.*, 1946, Pt. II, 5, 172.

³ Crescitelli, F., Koelle, G. B., and Gilman, A. F., *J. Neurophysiology*, 1946, 9, 241.

⁴ Rothberg, M. A., and Nachmansohn, D., *Fed. Proc.*, 1946, Pt. II, 5, 199.

⁵ Bullock, T. H., Grundfest, H., Nachmansohn, D., Rothberg, M. A., and Sterling, K., *J. Neurophysiology*, 1946, 5, 253.

Method. We have modified the procedures as follows. Both sciatics of a frog were immersed in peanut oil, containing the desired concentration of DFP. The nerves rested on stimulating and lead-off electrodes and were tested in place. When desired, to test reversibility, the oil was well washed out of the chamber and fresh oil substituted. Normally, after 3 hours one nerve was removed from the DFP solution for Ch.E. assay, the other left in and its action potentials followed at intervals until the conclusion of the assay. Maximal stimuli (tested repeatedly during an experiment) were used at 20/sec. during the test.

To obviate any possible objection to the CO₂ method, Ch.E. was determined by a direct measure of the A.Ch. at appropriate times after mixing an A.Ch. solution with the homogenized nerve extract. Controls showed that peanut oil traces were without influence on rate of hydrolysis. A.Ch. was assayed on the frog's rectus with the usual precautions. Typical conditions were: 20 to 50 mg of nerve homogenized in ½ cc of Ringer, added to 4.5 cc of 4.5×10^{-4} M A.Ch. in Ringer, assayed after ½, 1, 2 and 3 hours at room temperature, approximately 20°C. At each time interval aliquots of the initial A.Ch. solution and of each of the nerve-A.Ch. suspensions were assayed for A.Ch. activity.[†] The initial solution as assayed was diluted to 0.5 γ A.Ch. Br. in 4 cc Ringer.

Results. In a preliminary series, nerves were followed in various concentrations of DFP until action potentials were abolished or no further change was observed in several hours. Fig. 1 shows a set of results. At DFP concentrations above 15 mM, the action potential progressively falls, during hours or minutes depending on the strength

[†] Comparison of known and unknown solutions at each test is important, especially with DFP present. This substance, added with boiled or unboiled nerve but not by itself, potentiates the muscle response to known A.Ch. With 45 mM DFP, the muscle response may be doubled; at 6 mM the potentiation is around 20%; at 3 mM no potentiation was observed. The basis of this action has not been adequately studied, but no further formation of A.Ch. is involved.

of the solution, to zero. Such a depressed or inactive nerve has never shown a sign of recovery when washed with fresh solvent. (On transfer from oil to Ringer return of potential occurred one time). At DFP concentrations below 15 mM, little depression of the action potential has been observed over a period of 2 hours; at 3 mM, none in over 6 hours.

In the critical experiments, DFP at concentrations of only 3 mM were used. Fig. 2 shows the practically unchanged action potential in a nerve left exposed to 3 mM for 5 hours while its fellow was being assayed for Ch.E. The latter, after 3 hours in the DFP, exhibited zero Ch.E. activity, as shown in Table I. That the enzyme was not in-

TABLE I.
A.Ch. (as % of original amount) Present After
3 hr Incubation with Ground Nerve.

Exp.	Normal	DFP-treated
1	0	90
2	0	100
3	0	100
4	0	100
5	4	100
6	14	100
7	31	86

activated only during homogenization, by DFP previously unable to penetrate, is shown by an experiment in which a DFP nerve and a control nerve were ground together (Table II). Ch.E. activity was more rather than less than the average of poisoned and unpoisoned.

TABLE II.

Material tested (after 3 hr incubation)	Muscle response* (arbitrary units)	
	Exp. A	Exp. B
Substrate alone	55	50
Substrate plus DFP-treated nerve	57	55
Substrate plus normal nerve	0	0
Substrate plus DFP and normal nerves	8	12

* Response is proportional to A.Ch. present.

Conclusions. Frog nerve, exposed to DFP in a concentration and for a time sufficient fully to inactivate its choline esterase, can still conduct impulses with entirely normal action potentials. Ch.E. is not essential to nerve conduction.

Platelet Fragility in Man.

LEWIS R. DAY. (Introduced by Henry N. Harkins.)

From the School of Medicine, Johns Hopkins University.

For many years it has been known that a certain strain of swine demonstrates an abnormality of blood coagulation similar to that found in human hemophiliacs. The genetics of this abnormality and its mechanism have been extensively studied.¹⁻³

Working with this strain of swine, Muhrer, Bogart and Hogan⁴ have developed a method of testing platelet fragility. It is the purpose of the present study to determine (1) whether the method is applicable to the determination of platelet fragility in man, and (2) whether human platelets react similarly to those of swine.

The method resembles that used for testing erythrocyte fragility. Platelet-rich plasma is obtained by moderate centrifugation of whole blood, drawn with special precau-

tions to minimize trauma to the formed elements. The blood is drawn through a petrolatum-lined No. 20 McCrae needle directly into cold paraffined centrifuge tubes containing sodium citrate. This plasma is added to various hypotonic saline solutions, recalcified, and the time of coagulation measured. It is assumed that the mechanism of coagulation is the classical 2-stage process postulated by most workers in the field, and that coagulation time is quantitatively related to the speed of thrombin formation, which is in turn directly dependent upon the amount of thromboplastin released by platelets. One assumes further that if platelet-rich plasma exposed to hypotonic solutions clots faster than normal plasma, this is due to a more complete lysis of platelets. Conversely, if 2 different specimens, after similar exposure to saline solutions of the same tonicity, have different clotting times, there is presumably a difference in platelet fragility.

Critique of Method. (1) To determine whether the effect of the hypotonic solutions was on some phase of the clotting mechanism other than the platelets, the specimens, after exposure to the various saline solutions, were all brought up to 0.9% NaCl, prior to recalcification. Under such circumstances, clotting occurred in a normal saline solution. As is shown in Fig. 1, the specimens exposed to hypotonic solutions clotted faster, even though clotting took place in a normal saline solution; and the more hypotonic the solution, the faster was the coagulation on eventual recalcification. The longer the time of exposure to the hypotonic solution, prior to recalcification, the more coagulation was accelerated. (Fig. 2). This suggests that, as in the case of erythrocytes, platelets do not disintegrate all at once, but are slowly lysed over a period of about 15 minutes.

Similar results were obtained with highly centrifuged specimens (Fig. 2). Although smears of these plasmas showed no cells, the

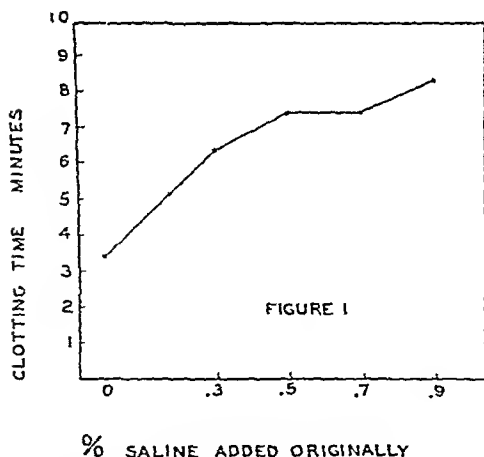


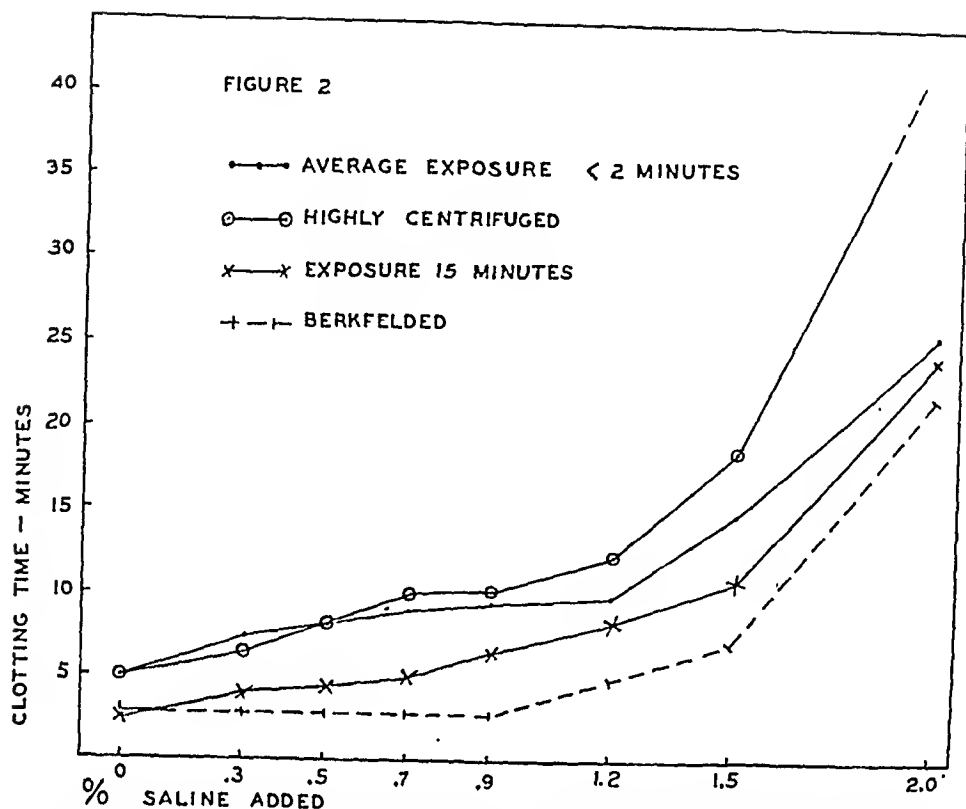
FIGURE 1
Effect of saline solutions on clotting time of normal platelet-rich plasma; reaction taking place in 0.9% saline.

¹ Hogan, A. G., Muhrer, M. E., and Bogart, R., *Proc. Soc. Exp. Biol. and Med.*, 1941, **48**, 217.

² Bogart, R., and Muhrer, M. E., *J. Hered.*, 1942, **33**, 59.

³ Muhrer, M. E., Hogan, A. G., and Bogart, R., *J. Physiol.*, 1943, **136**, 355.

⁴ Muhrer, M. E., Bogart, R., and Hogan, A. G., *Am. J. Physiol.*, 1944, **141**, 449.



Effect of saline solutions on clotting time of normal platelet-rich, platelet-poor, and platelet-free plasma and the effect of varying the exposure time; reactions taking place in solutions of varying tonicity.

results were essentially the same. However, it must be assumed that they contained platelets, since when specimens were passed through a Berkefeld N filter, exposure to hypotonic solution no longer affected clotting time significantly (Fig. 2).

These data suggest that the method as described by Muhrer does test the resistance of the platelets to hypotonic saline solution.

Platelet Fragility in Man. (2) The results with several normal human specimens are shown in Fig. 3. These were taken from normal healthy medical students and from white male patients, all of whom had apparently normal clotting mechanisms.

Specimens were also drawn from a known hemophiliac who is 40 years old and has been treated at the Johns Hopkins Hospital since the age of 5. These results are shown in Fig. 3. The curves are similar to those obtained in hemophilic swine, the difference

being that in the latter the clotting time was reduced to normal levels by exposure to distilled water, while in man the clotting time was shortened, but not to normal levels.

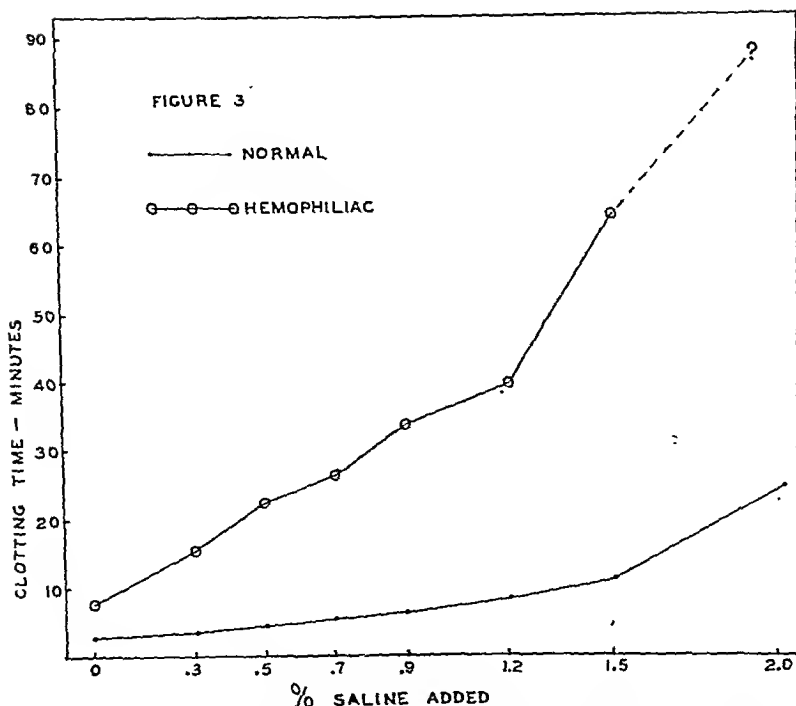
Discussion. Extensive studies of the clotting mechanism in normal humans and hemophiliacs to determine the fundamental abnormality have evolved several theories.⁵⁻⁸ The present study on the resistance of normal and hemophilic platelets is offered as additional but not conclusive evidence that the difference between hemophilic and normal blood clotting lies in the platelets and not the plasma. Further study is necessary,

⁵ Minot, G. R., and Lee, Roger I., *Arch. Int. Med.*, 1916, **18**, 474.

⁶ Howell, W. H., and Cekada, E. B., *Am. J. Physiol.*, 1926, **78**, 500.

⁷ Patek, A. J., and Stetson, R. P., *J. Clin. Invest.*, 1936, **15**, 531.

⁸ Kark, R., *Clinics*, 1943, **2**, 15.



Effect of saline solutions on clotting time of platelet-rich plasma in hemophilia as compared with normal; reactions taking place in solutions of varying tonicity.

however, for it is entirely possible that the increased lysis of platelets by supplying an excess of thromboplastin serves to counteract some other deficiency or excess in hemophilia.

As suggested by Muhrer, one of the chief drawbacks in the testing of substances active in reducing clotting time in hemophilia is the lack of suitable experimental animals. These findings with human blood are sufficiently

close to those obtained with the blood of hemophilic swine to suggest the possibility that study of the latter may throw light on the human disease.

I am indebted to Dr. Philip Wagley of the Johns Hopkins Hospital and to Dr. Harry Eagle of the United States Public Health Service for technical aid and advice in carrying out this study.

Present address: Presbyterian Hospital, Chicago.

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Effect of 2-Methyl-1,4-Naphthoquinone on Glycolysis of *Schistosoma mansoni*.*

ERNEST BUEDING, LAWRENCE PETERS, AND JEAN F. WAITE.

From the Department of Pharmacology, Western Reserve University School of Medicine, Cleveland.

Earlier observations indicated that oxidative metabolism is not essential for the sur-

vival of *Schistosoma mansoni*.¹ Since schistosomes have a high rate of both anaerobic

* The work described in this report was done under a contract between the Office of the Surgeon General, U. S. Army and Western Reserve Uni-

versity.
¹ Peters, L., Welch, A. D., and Bueding, E., unpublished data.

TABLE I.
Effect of Quinones on Oxygen Uptake and Glycolysis of Schistosomes.

Compound	Molar concn.	Medium	O ₂ uptake		Glucose removal		Lactic acid production	
			μl^*	% change	μg^*	% change	μg^*	% change
2-Methyl-1,4-naphthoquinone	1x10-4	Salt	1.21	-51	33.5	-100	30.4	-76
1,4-Naphthoquinone	2x10-5	"	0.59	+3	0	-36	7.2	-24
1,2-Quinone	2x10-5	"	1.25	-21	21.6	-58	23.2	-40
1,4-Quinone	2x10-4	"	0.94	-5	13.6	-13	18.1	-7
	2x10-5	"	1.15	+4	28.9	-26	28.0	-11
1,4-Hydroquinone	2x10-4	"	1.26	-22	24.8	-79	27.1	-48
	2x10-5	"	0.94	-6	7.0	-16	16.1	-7
3-Methoxy-2-methyl-1,4-naphthoquinone	2x10-4	"	1.18	-28	28.2	-50	28.3	-32
3-Hydroxy-1,4-naphthoquinone†	1x10-3	"	0.87	-38	16.7	-25	20.9	-65
3-Hydroxy-2-piperidino-methyl-1,4-naphthoquinone†	1x10-3	"	0.51	-52	16.7	-35	13.6	-27
3-Hydroxy-2-amylo-amino-methyl-1,4-naphthoquinone†	1x10-3	"	0.58	-34	25.3	-72	22.3	-27
	1x10-3	"	1.19	-34	29.4	-35	27.4	-27
2-Methyl-1,4-naphthoquinone	5x10-4	Buffered serum	0.80	-24	21.9	-87	22.3	-69
	1x10-4	"	1.40	+2	37.6	-37	44.6	-30
1,4-Naphthoquinone	1x10-4	"	1.06	-16	4.9	-25	13.7	-18
Quinone	2x10-3	"	1.42	-	23.6	-68	31.3	-55
Hydroquinone	2x10-3	"	1.18	-	28.3	-46	36.5	-22
	2x10-3	"	-	-	12.0	-	20.2	-
	2x10-3	"	-	-	21.4	-	34.9	-

* Per hour and mg wet wt.

† Kindly supplied by Abbott Laboratories, Chicago.

and aerobic glycolysis, studies have been made of the glycolytic processes and of their inhibition by compounds of low toxicity for mammalian species.

Paired adult schistosomes were removed from the mesenteric or portal veins, or from the larger hepatic vessels of mice infested with *S. mansoni*. The organisms were placed in a glucose-containing salt medium (0.137 *M* NaCl, 0.0027 *M* KCl, 0.0003 *M* CaCl₂, 0.001 *M* MgCl₂, 0.066 *M* phosphate; pH, 7.4) or in a mixture of 40 volumes of human serum (previously degassed and adjusted to pH 7.4) and 3 volumes of 0.5 *M* phosphate buffer (pH 7.4). A high phosphate concentration is essential for the optimal respiration and glycolysis of schistosomes *in vitro*. Incubation was then carried out in small vessels (vol. 4 to 5 ml) in 0.8 ml of medium at 38.6°C in an atmosphere of air; the consumption of oxygen was measured manometrically. The concentrations of glucose² and of lactic acid³ in the medium were determined before and after the incubation period.

The rate of aerobic glycolysis of schistosomes was inhibited by 2-methyl-1,4-naphthoquinone (representative results are recorded in Table I). Oxygen uptake was inhibited to a much smaller extent than was the removal of glucose or the formation of lactic acid. The concentration of 2-methyl-1,4-naphthoquinone required to produce a 50% inhibition of glycolysis was about 5 times greater in human serum than in a protein-free salt medium. If schistosomes were incubated for 30-60 minutes at 38°C in the serum-medium, containing the naphthoquinone in a concentration of 1×10^{-4} *M*, and were then transferred into fresh medium without the quinone, inhibition of glycolysis was maintained for a period of at least 4 hours. As recorded in Table I, a related compound, 1,4-naphthoquinone, was somewhat more effective than the 2-methyl derivative in inhibiting glycolysis of schistosomes in a salt medium, but it was less effective in ser-

um. 1,2-Naphthoquinone was a much weaker inhibitor of glycolysis of schistosomes than was 1,4-naphthoquinone or 2-methyl-1,4-naphthoquinone. Furthermore, 3-methoxy-2-methyl-1,4-naphthoquinone[†] and substituted 3-OH-naphthoquinones were much less potent inhibitors of glycolysis of *S. mansoni* than was 1,4-naphthoquinone or 2-methyl-1,4-naphthoquinone. It appears, therefore, that introduction of a hydroxy- or a methoxy-group in position-3 decreases markedly the activity of a naphthoquinone against schistosomes *in vitro*.

When mice infected with schistosomes were fed a diet containing 1 to 1.5% 2-methyl-1,4-naphthoquinone for one week and, during this period, were injected intraperitoneally with subcurative doses of sodium antimony III-biscatechol-2,4 disulfonate ("Fuadin") (7.5 mg/kg every 8 hours for 5 days), the schistosomes removed from the livers of these mice showed a markedly decreased rate of glycolysis, as indicated by 60 to 90% reduction of glucose removal and of lactic acid formation. Furthermore, the combined administration of the antimonial compound and the quinone resulted in the complete disappearance of the schistosomes from the mesenteric veins and in many cases from the portal vein of the host. The administration of 2-methyl-1,4-naphthoquinone alone, or of "Fuadin" alone, resulted in a much smaller decrease in the glycolytic activity (10-20%) of the parasites and did not lead to the exodus of the worms from the mesenteric veins.

Conclusions. 2-Methyl-1,4-naphthoquinone markedly inhibits aerobic glycolysis of *Schistosoma mansoni* *in vitro*. Since glycolysis, rather than oxidative metabolism, appears to be essential for the survival of these organisms, and since the toxicity of 2-methyl-1,4-naphthoquinone for mammalian species is low, the effect of this compound in experimental schistosomiasis has been studied. Results in mice suggest that the compound may act synergistically with subcurative doses of antimonials ("Fuadin").

² Somogyi, M., *J. Biol. Chem.*, 1945, **160**, 61, 69.

³ Barker, S. B., and Summerson, W. H., *J. Biol. Chem.*, 1941, **138**, 535.

[†] Kindly supplied by Dr. C. Colwell of The Velsicol Corporation, Chicago.

A Practical Method for Routine Blood Cultures in Brucellosis.

M. RUIZ CASTANEDA.

From the Department of Medical Research, Hospital General, Mexico, D.F.

Various methods have been used in our laboratory for the isolation of *Brucella* organisms from the blood of patients. Only a minor proportion of the cultures are positive since we are dealing with unselected cases which include patients with other diseases; and because on the brucellosis cases serial blood cultures are performed during the course of the disease. The time-consuming bacteriological procedure and the waste of material entailed in such a large percentage of negative cultures made it advisable to look for other dependable methods for the detection of positive blood cultures avoiding troublesome secondary bacteriological transplants. The method as devised also minimizes the handling of possibly infective material, since such handling has proved dangerous to the technician who sooner or later becomes infected with the melitensis strain. Various procedures were investigated and the one described below has given the most satisfactory results.

The medium used is prepared with bactotryptose. The formula follows:

Bactotryptose	2.0 g
Sodium chloride	0.5 "
Sodium citrate	0.5 "
Agar	3.0 "
Distilled water	100 ml

Fifteen ml of the medium are placed in 100 ml flat-sided, rectangular bottles. The mouth of the bottle (about $1\frac{1}{4}$ cm in diameter) is covered with thick paper and sterilized at 15 lb for 15 minutes. The bottles are placed on their sides so that the agar sets on one of the narrow sidewalls forming an even, transparent layer. The paper is then lifted, using aseptic technic, and 10 cc of bactotryptose broth are added. The flask is then closed with sterile rubber stoppers and the bottle again covered with the same paper cap. The broth contains 2% bactotryptose and 2% sodium citrate and is sterilized in separate containers at 15 lb for 15 minutes. The air within the bottles can be replaced by suitable mixtures of CO₂ and air by any mechanical

device which avoids contamination of the double medium.

Before using this double medium, tests for sterility are made at 37°C for 3 or 4 days. The agar surface is wet by the broth at 24-hour intervals.

The double medium may be inoculated with 10 cc of blood, the patient usually being bled with a 20 cc autoclave-sterilized syringe. The needle is inserted through the rubber stopper after the careful removal of the paper cap. The mixture of blood and broth is spread over the agar layer for a moment and the bottle incubated in a vertical position at 36°C. Every other day the broth is allowed to flow over the agar layer and the bottle replaced in the incubator, again in a vertical position.

Results. When the agar layer shows colonies after 24-48 hours, it is likely that the culture has been contaminated. When the colonies appear 24 or 48 hours after the second inoculation, the cultivated organism has usually been found to be a *Salmonella*, less frequently *Staphylococcus* or *Streptococcus*, but may be *Brucella*. Positive cultures of *Brucella* are detected more frequently after the third agar inoculation, that is on the sixth day of cultivation. After 20 days the negative cultures may safely be discarded. The number of colonies and time of their appearance on the agar layer vary from one case to another and serve as a good indication of the intensity of the bacteriemia. Serial cultures performed twice a month have been of assistance in roughly estimating the course of the bacteriemia. During the acute phase of the disease the early appearance of innumerable colonies is the usual finding. In the chronic phase when positive, the culture shows few or sometimes a single colony in 10 or more days.

The colonies of *Brucella* are clearly seen through the agar layer and are easily transferred for further study. With some experience one may differentiate them from contaminants, *Salmonella* and other organisms.

We consider that the ease with which posi-

tive cultures are detected and the reduction of bacteriological manipulation and material, plus the increased safety to the technician, compensate for the trouble taken in the preparation of the double medium. Furthermore, the technician in charge of identification of the organism receives only positive cultures and need not be warned about careless handling of the material. The possibilities of accidental infection are thereby reduced. The increase in the proportionate amounts of agar in the solid medium is necessary in order to prevent the deterioration of the agar layer. The rather high citrate content is intended not only to prevent clotting of the blood but also to reduce the phagocytic activity of polymorpho-

nuclear leucocytes to a minimum. It is thought that the ease with which the *Brucella* colonies appear on the agar layer is due at least in part to the absence of plasma, thus reducing the bactericidal effect.

The 100 cc bottles have been found suitable for inoculation with 100 ml of blood. However, 200 or even 500 ml bottles may be used if one desires to culture larger amounts of blood.

Summary. A double medium for blood cultivation in brucellosis is described. The method saves time and material by avoiding transfers and affords increased safety against accidental infection to the personnel.

15718

Hematologic Effects of Pteroylglutamic Acid (Folic Acid) in Man.*

GRACE A. GOLDSMITH. (Introduced by John H. Musser.)

From the Department of Medicine, Tulane University School of Medicine, New Orleans, La.

Pteroylglutamic acid, commonly known as folic acid or *L. casei* factor, has been shown by several groups of investigators to be an effective hematopoietic substance in human macrocytic anemia.^{1,2,3} Since 1945 when synthetic pteroylglutamic acid became available, 23 patients with macrocytic anemia have been treated with this material in our clinic. The findings obtained in 15 patients during the first few months of therapy have been reported.^{4,5,6} Five patients have now been fol-

lowed for approximately one year and 2 additional patients for over 6 months. In each instance, the clinical and hematological improvement which followed the administration of pteroylglutamic acid has been maintained.

Seventeen of the 23 patients studied were benefited by therapy. Hematologic findings at the initial and most recent visits to the clinic, and the amount and method of administration of pteroylglutamic acid are given in Table I. In all patients with pernicious anemia and nutritional macrocytic anemia there was a marked increase in the erythrocyte count, in hemoglobin and in the volume of packed red blood cells. In patients with sprue the blood picture was affected in only 3 of 5 cases. However, each patient improved in other respects including relief of glossitis and diarrhea, gain in weight, better absorption of nutrients from the intestinal tract as indicated by the glucose tolerance test, and diminution in steatorrhea. In one patient with cirrhosis of the liver there was a slight increase in the blood count after pteroylglutamic acid was administered while in 5 patients with macrocytic anemia of varied

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¹ Spies, T. D., *J. Am. Med. Assn.*, 1946, **130**, 474.

² Darby, W. J., Jones, Edgar, and Johnson, N. C., *J. Am. Med. Assn.*, 1946, **130**, 780.

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⁵ Goldsmith, G. A., *Science*, 1946, **104**, 439.

⁶ Goldsmith, G. A., *J. Lab. and Clin. Med.*, 1946, **31**, 1186.

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TABLE I.
Effect of Pteroylglutamic Acid on the Blood Picture in Macrocytic Anemia.

Pteroylglutamic Acid on the Blood Picture in Macrocytic Anemia.										
Sex Race	Hematologic findings						No. months observation	Pteroylglutamic acid therapy		
	Initial			Final				mg/day	Time	Route
	RBC	Hb	Hct	RBC	Hb	Hct				
WF	1.7	6.2	21	4.2	13.0	40	12	15‡	21D	IM
CM	1.7	4.9	14	4.0	13.0	44	12	15	7M	O
								5	3M	O
								15	21D	IM
								0	6M	—
WM	3.5	10.5	36	5.0	14.9	46	7	5	5M	O
								120	5D	O
								30	14D	O
CM	1.0	3.3	10	3.5	11.2	41	2	5	6M	O
WF	1.4	6.0	21	4.2	13.2	42	3.5	5	2M	O
WF	2.1	8.2	23	4.6	14.5	47	3.5	10	3M	O
CM	1.5	4.2	16	4.0	10.3	39	2	10	3.5M	O
								5	2M	O
Nutritional Macrocytic Anemia.										
WF	1.8	6.0	20	3.9	12.0	39	12	15‡	21D	IM
								15	3M	O
								30	1M	O
								5	6M	O
WF	2.3	7.0	22	4.1	13.9	44	11	15‡	21D	IM
								30	2M	O
								5	6M	O
WF	2.3	9.5	25	4.0	12.3	39	13	5-15‡	5M	O
								30	3M	O
								5	5M	O
WF	2.3	8.4	29	4.4	11.5	40	8	40‡	2M	IM
								100‡	7D	O
								5	5M	O
Sprue.										
WF	3.5	11.5	39	3.7	10.2	35	3.5	50	21D	O
								20-40‡	2M	IM
WF	3.6	11.3	38	3.9	11.9	39	2.5	30	2M	O
WM	2.1	7.3	27	4.0	12.5	39	3	5	3M	O
WM	3.0	10.0	36	3.4	11.0	37	1	20‡	1M	IM
WF	3.4	10.9	37	3.8	11.5	38	1	10	1M	O
WM*	3.9	10.7	38	4.4	12.6	41	1	20	14D	IM

IM—Intramuscular administration; O—Oral administration; D—Days; M—Months; Hct—Hematocrit; volume of packed erythrocytes %.

* Celiac disease.

† Therapy intermittent.

‡ Therapy omitted for 1-2 months after this period of treatment.

§ This dosage administered 3 times weekly.

¶ This dosage administered every 4 days.

etiology there was no appreciable change (2 had aplastic anemia, one each, anemia associated with regional ileitis and myxedema and one anemia of unknown origin).

The hematologic effects of pteroylglutamic acid closely resembled those obtained with liver extract. Within 2 to 6 days after therapy was instituted the percentage of reticulocytes in the peripheral blood increased, a maximum rise being attained in 5 to 14 days. When pteroylglutamic acid was given orally the increase in reticulocytes was similar to that

which is usually observed when optimal amounts of liver extract are administered; when given parenterally the increase in reticulocytes was usually less than this (Fig. 1). During the first few days of treatment hemodilution occurred and edema was found on physical examination. The erythrocyte count, percentage of hemoglobin and the volume of packed red blood cells began to rise 7 to 10 days after therapy was instituted. The increase was rapid during the first month and then gradual until normal or slightly sub-

Reticulocyte Response to Pteroylglutamic Acid

administered orally and parenterally
to patients with macrocytic anemia

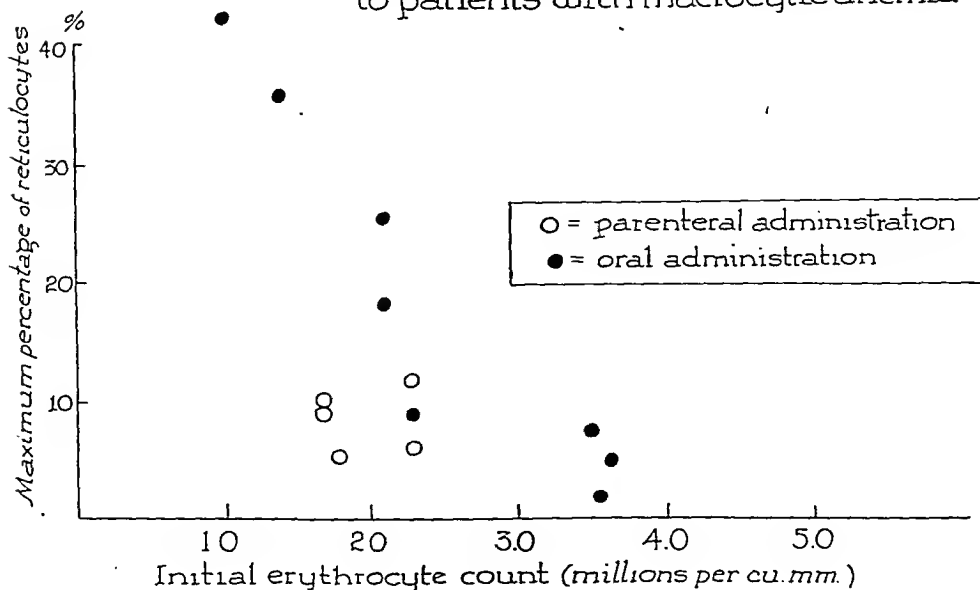


FIG. 1.

The amount of pteroylglutamic acid given parenterally was 15 mg or more daily; the oral dose in the four patients with a reticulocyte response above 15% was 5 to 10 mg daily.

normal levels were reached. The monthly erythrocyte counts for 7 patients who have been followed for over 6 months are given in Table II. The maximum rise occurred by the third month in most instances and this level has been maintained essentially unchanged.

The count has remained below 4.5 million in a majority of the patients in spite of intensive treatment. This may indicate a difference between the effects of pteroylglutamic acid and of liver extract.

A dose of 5 mg of pteroylglutamic acid daily

TABLE II.
Erythrocyte Count in Patients with Macrocytic Anemia During Treatment with Pteroylglutamic Acid.

Sex Race	Initial count	Erythrocyte count (millions per mm ³) monthly intervals												
		1	2	3	4	5	6	7	8	9	10	11	12	13
		Pernicious Anemia.												
W.F.	1.72	3.20	3.32	4.0	3.9	4.05	4.3	3.9	4.5	4.51	3.4	3.6	4.2	
C.M.	1.72	3.30	3.5	4.1	4.2	3.0	2.5	2.31	3.4	3.5	4.3	4.2	4.0	
W.M.	3.44	4.21	5.8	6.0	4.4	4.5	4.7	5.0						
		Nutritional Macrocytic Anemia.												
W.F.	1.82	3.40	3.32	4.0	3.9	3.93	4.21	4.2	4.3	3.9	4.0		3.9	
W.F.	2.32	3.50	3.6	3.63	3.7	3.81	4.4	4.5	4.5	4.3	4.0	4.1		
W.F.*	2.31	2.92	3.00	3.12	3.40	3.93	4.0	4.1	4.21	4.0	4.0	3.8	4.0	4.0
W.F.	2.36	3.7	3.77	4.01	4.2		4.0	4.1	4.4					

* Therapy intermittent during first 5 months.

Amount of pteroylglutamic acid administered and changes in therapy are indicated by numerals as follows: (0) no therapy; (1) 5 mg daily; (2) 15 mg daily; (3) 30 mg daily; (4) 120 mg daily for 5 days then 30 mg daily for 2 weeks; (5) iron added to therapy; (6) 40 mg three times weekly; (7) 100 mg daily for 1 week then none for 1 month.

appeared to be approximately as effective as were larger amounts in producing reticulocytosis and an increase in erythrocyte count. The administration of 30 mg daily did not cause significant improvement over that seen with 15 mg a day (cases 4-5-6, Table II). However, the only patient (Case 3) whose red cell count reached 5 million was given very large amounts of pteroylglutamic acid initially. The 7 patients who have been followed for prolonged periods have received only 5 mg daily for the past 3 to 6 months. During this time the erythrocyte counts have remained essentially constant. This dosage seemed to be sufficient and may be more than the minimal amount needed for maintenance.

The initial leukocyte count was low (less than 3500 per mm³) in 6 patients included in this study. In each instance there was a return to normal when pteroylglutamic acid was administered and the percentage of granulocytes increased. The rise in white cells began during the first week of therapy and reached a maximum in 1 to 3 weeks.

The bone marrow was studied in most of

the patients treated with pteroylglutamic acid and showed a rapid return to normal. In pernicious anemia the marrow is hyperplastic, megaloblasts being the predominant cell. In one patient in whom serial studies were undertaken, megaloblasts had decreased in number at the end of 5 days and were rarely observed at the end of 2 weeks. In patients with nutritional macrocytic anemia the marrow was at times hyperplastic and at others hypoplastic. The predominant cell was the megaloblast in some instances, the normoblast in others. Regardless of the findings prior to therapy, the bone marrow became normal within 4 to 6 weeks.

Conclusion. The administration of pteroylglutamic acid was followed by definite improvement in 17 of 23 cases of human macrocytic anemia. The hematologic effects of pteroylglutamic acid closely resembled those of liver extract but differed in that oral administration was equally or more effective than parenteral and that restoration of the blood picture to normal was often incomplete.

15719 P

Antibiotic Activity of Certain Molds Against *Brucella*.*

GRACE A. BEAL.[†] (Introduced by S. A. Koser.)

From the Department of Medicine and the Department of Bacteriology and Parasitology, The University of Chicago.

Although a number of mold antibiotics have been described,¹ few reports indicate activity against *Brucella spp.*^{2,3} The ineffectiveness of penicillin against bacteria of this group was early recognized,⁴ and this observation has received repeated confirmation. Despite the demonstration of inhibition of *Brucella* by high concentrations of penicillin,^{5,6} resistance of these organisms is so great that the drug

has been successfully used in concentrations of 2-5 Oxford units per ml for selective isolation of *Brucella* from material containing other more sensitive bacteria.⁶

Experimental. In a preliminary study 202 mold cultures,[†] including strains of *Aspergillus*, *Penicillium*, *Absidia*, *Rhizopus*, *Mucor*, *Chaetomium*, *Trichoderma*, *Sporotrichum*,

* T'ung, Tsun, *PROC. SOC. EXP. BIOL. AND MED.*, 1944, 50, 8.

† Fleming A., *Second Int. Congress for Microbiology*, Report of Proceedings, London, 1936, 33.

5 Hobby, Gladys L., *Science*, 1944, 100, 500.

6 Beal, G., unpublished data.

* Aided by a grant from Swift and Company.
† The early part of this study was made at The Brucellosis Research Laboratory, Clayton Foundation, The University of Texas.

1 Waksman, Selman A., *Microbial Antagonism and Antibiotic Substances*, The Commonwealth Fund, 1945.

2 Kocholaty, Walter, *J. Bact.*, 1942, 44, 469.

† Cultures were kindly made available by Dr. M. B. Morrow, Department of Botany and Bacteriology, The University of Texas.

TABLE I.
Antibiotic Activity of Certain Molds Against *Brucella* and *Staphylococcus*.

Mold culture	Source of mold	Activity of Filtrates*	
		<i>Brucella abortus</i> 295	<i>Staphylococcus aureus</i> 209
<i>Aspergillus terreus</i> No. 1	Stock collection	+	0
" " No. 2	" "	+	0
" " No. 3	" "	+	0
" " No. 4	" "	+	+
" <i>sydowii</i> No. 1	" "	0	+
" <i>nidulans</i> No. 1	" "	0	+
<i>Penicillium carminoviolaceum</i> No. 1	" "	+	+
" <i>sartoryi</i> No. 1	" "	+	+
" <i>chrysogenum</i>	" "	0	+
" sp., SD-1	Soil†	+	+
" " SD-21	"	+	+
<i>Aspergillus glaucus</i> , SD-3	"	+	+
" <i>versicolor</i> , SD-5	"	0	+
" <i>terreus</i> , SD-16	"	+	+
" " SD-17	"	+	+
" <i>fumigatus</i> , SD-19	"	0	+
" <i>flavipes</i> , SD-16	"	+	+
" <i>humicola</i> , SD-23	"	+	0
" <i>versicolor</i> , SD-7	"	0	+

* Filtrates of mold cultures in Czapek-Dox medium were tested against 0.1 ml of 1:10 dilution of bacterial culture in 100 ml tryptose—0.1% dextrose agar. + = inhibition; 0 = no inhibition.

† Freshly isolated from lot inclosing *Brucella*-infected cattle.

Pullularia, Hormodendrum, Curvularia, Helminthosporium, Stemphylium, Alternaria, Spondylocadium and Fusarium, were tested for inhibition of *Br. abortus* 295 (Huddleson) and *Staph. aureus*. Molds were grown at room temperature in a tryptose-0.1% dextrose broth, pH 7.3. Metabolite samples, diluted in Locke's solution, were tested for activity after 6, 9, 12, 19, and 26 days against *Staph. aureus* and *Br. abortus* by a modified cup method,⁷ using 20 ml of tryptose agar seeded with 0.1 ml of a 24-hr culture of the test bacteria. One culture of *Penicillium spinulosum* inhibited growth of *Staph. aureus* and one *Aspergillus flavus* inhibited both *Staph. aureus* and *Br. abortus* by this method.

Thirty-two strains of *Penicillium* and *Aspergillus* found to be inactive in the above tests were grown in modified Czapek-Dox medium⁸ and the undiluted filtrates were assayed against *Br. abortus* 295 and *Staph. aureus* 209,

using 0.1 ml of 1:10 dilution of bacterial culture in 100 ml tryptose agar. Filtrates of cultures of nine of the molds were active against one or both of the bacteria. Of 24 molds isolated from soil of an inclosure in which *Brucella*-infected cattle were kept, 10 were found to be active by the same method (Table I). In some instances activity was slight or inconsistent.

Mold SD-17, tentatively classified as *Aspergillus terreus*, was particularly active against *Brucella*. On Czapek-Dox medium this culture produces a tan-colored colony which is brown on the reverse side; microscopically the heads are columnar, and the conidia smooth and globose. Further studies were made on cultures of this mold grown in Czapek-Dox broth. Active filtrates of a 14-day culture were wine-colored. The active material is found in the ether soluble fraction, is heat stable (boiling 10 minutes), and after partial purification inhibits *Br. abortus in vitro* in a dilution of 1:64,000.

Since citrinin is produced by a related species,⁹ comparative studies have been made with crystalline citrinin supplied by Dr.

⁷ Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., Jennings, M. A., and Florey, H. W., *Lancet*, 1941, 2, 177.

⁸ Hobby, Gladys L., Meyer, Karl, and Chaffee, Eleanor, *Proc. Soc. Exp. Biol. and Med.*, 1942, 50, 277.

⁹ Timonin, M. I., *Science*, 1942, 96, 494.

Timonin. The antibacterial spectra of the two substances are similar and the activity of mold SD-17 may be due to citrinin. The active material produced by mold SD-17 has not been obtained in a crystalline form, and its identity has not been determined.

Summary. Filtrates of 13 mold cultures

were found to possess antibiotic activity against *Br. abortus in vitro*. The active material from one of these molds, tentatively identified as *Aspergillus terreus*, after partial purification, inhibited growth of *Br. abortus* in a dilution of 1:64,000.

15720

Effect of Para-Aminohippurate on Mannitol Determinations by the Periodate-Iodide-Thiosulfate Method.*

HAROLD G. BARKER[†] AND JOHN K. CLARK.[‡] (Introduced by C. F. Schmidt.)

From the Laboratory of Pharmacology, University of Pennsylvania, Philadelphia.

In using the combination of mannitol¹ and para-aminohippurate² in renal function studies we noticed discrepancies which suggested to us that the presence of para-aminohippurate (PAH) might be affecting the chemical analyses for mannitol. Therefore, we prepared solutions containing no mannitol but in which known amounts of free para-aminohippuric acid (PAHA) were added in varying concentrations to water and to sugar- and protein-free filtrates of human plasma and of urine. These solutions were then analyzed by the technic used for estimation of mannitol.¹ This involves oxidation of mannitol with a known excess of KIO_4 and determination, by titration with thiosulfate, of the remaining KIO_4 together with the KIO_3 produced in the reaction. Fig. 1 shows the results of these analyses. It can be seen that for every mg % of free PAH present, there is an apparent mannitol concentration of from 0.25 to 0.30 mg %.

* The expenses of this investigation were defrayed in part by a grant from the Life Insurance Medical Research Fund to the Department of Pharmacology, University of Pennsylvania.

† Research Fellow in Pharmacology and Fellow in the Harrison Department of Surgical Research.

‡ Research Fellow in Pharmacology.

¹ Smith, H. W., Finkelstein, N., and Smith, H. W., *J. Biol. Chem.*, 1940, **135**, 231.

² Smith, H. W., Finkelstein, N., Aliminosa, L., Crawford, B., and Graber, M., *J. Clin. Invest.*, 1945, **24**, 388.

In order to illustrate the extent to which this error can be expected to influence the various data of clinical interest, in a representative renal work-up with mannitol and PAH, we have prepared Table I. The data are calculated for an hypothetical patient based on a plasma mannitol level of 1 mg per cc throughout; a plasma PAH level of 0.02 mg per cc during the "plasma flow period" and 0.75 mg per cc during the "Tm period." The glomerular filtration rate is taken as 130 cc per min., the effective renal plasma flow as 670 cc per min., and the Tm_{PAH} as 75 mg per min. It is assumed that there is a false elevation of 0.27 mg % in mannitol levels for each mg % of PAH present.

One study in the literature³ reports the glomerular filtration rate (mannitol) on each of 2 different days (4 in 2 cases) in 23 patients. In each instance, the mannitol clearance was done during a Tm determination with PAH on one day and during a diodrast Tm on the other day. In the 25 pairs, the mannitol clearance was higher on the PAH day in 21 cases and lower in 4 instances. The mean C_{M} was 9.2% higher when PAH was present at Tm levels than on days when diodrast was used. This agrees well with the 10% expected error listed in Table I.

³ Chasis, H., Redish, J., Goldring, W., Ranges, H. A., and Smith, H. W., *J. Clin. Invest.*, 1945, **24**, 583.

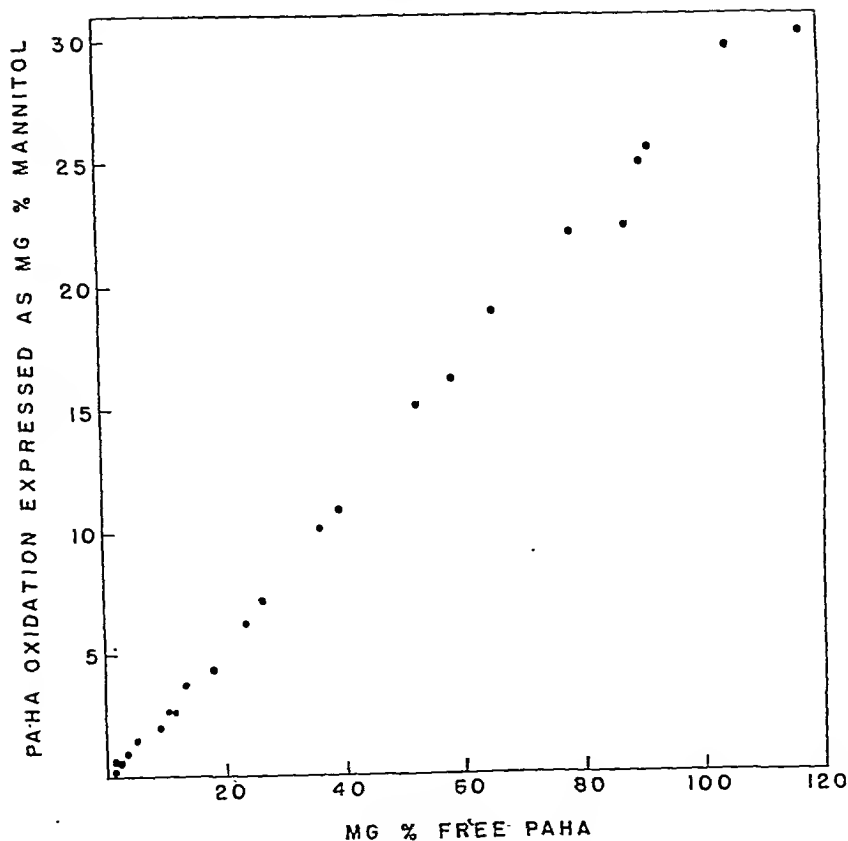


FIG. 1.

Para aminohippuric acid solutions analyzed by the unmodified method used for mannitol determination.

In the same paper an attempt was made to establish the Tm_{PAH} for normal subjects. One series of 10 cases gave a mean Tm_{PAH} of 67.6 using mannitol to determine glomerular filtration rate and another series of 21 cases

gave a mean of 82.2 using inulin for filtration rate estimation. It would seem that the normal Tm_{PAH} is probably higher than 77.5 (the average for the 2 series) and the standard deviation is less than 12.9, as there

TABLE I.

Errors in Clinical Data to Be Expected from the Unmodified Mannitol Method When Used in the Presence of PAH.

	Correct	Apparent		% error	
		During C_{PAH}	During Tm_{PAH}	During C_{PAH}	During Tm_{PAH}
Mannitol excretion ($U_M V$) mg/min	130	134	173	3	30
Plasma mannitol level (P_M) mg/cc	1	1,006	1.2	0.6	20
Glomerular filtration rate (C_M) cc/min	130	133	143	2	10
Max. tubular excretory capacity (Tm_{PAH}) mg/min	75		67		10
Filtration fraction (C_M/C_{PAH})	0.194	0.20		3	
Filtration rate per functioning unit (C_M/Tm_{PAH})	1.9		2.6		40
Effective renal plasma flow per functioning unit (C_{PAH}/Tm_{PAH})	8.9		10		10

See text for plasma levels, etc., assumed in preparation of this table.

Timonin. The antibacterial spectra of the two substances are similar and the activity of mold SD-17 may be due to citrinin. The active material produced by mold SD-17 has not been obtained in a crystalline form, and its identity has not been determined.

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15720

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¹ Smith, W. W., Finkelstein, N., and Smith, H. W., *J. Biol. Chem.*, 1940, **135**, 231.

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³ Chasis, H., Redish, J., Goldring, W., Ranges, H. A., and Smith, H. W., *J. Clin. Invest.*, 1945, **24**, 583.

Factors Not Synthesized by Yeast in the Presence of Cyanide.

MARGARET A. EPPRIGHT* AND ROGER J. WILLIAMS.

From the Clayton Biochemical Institute and the Department of Chemistry, University of Texas, Austin.

This study was undertaken as a result of difficulties encountered in growing yeast in a synthetic medium in the presence of potassium cyanide.¹ Other investigators^{2,3} have reported no difficulty in culturing *Saccharomyces cerevisiae* in a medium containing the same concentration of cyanide, but their media contained malt extract or yeast extract. Presumably the extracts supply one or more substances which the yeast is unable to synthesize with sufficient rapidity in the presence of cyanide. Direct experiments supported this view.

A method for quantitative study of the active principle or principles present in yeast extract was devised, the procedure being patterned after that used in the assay method for biotin.⁴ Initially, the only modification in the culture medium was the addition of a sufficient amount of potassium cyanide to give a concentration of 0.001 M. Later, pantothenic acid (2 mg per liter) was substituted for β -alanine, and cysteine (20 mg per liter) was added as a supplement. The standard of activity was Difco yeast extract which was added over a range of 0 to 10 mg. The test organism was *Saccharomyces cerevisiae*, F.B., and the extent of growth was measured turbidimetrically after an incubation period of 15 to 16 hours at 30°C. Table I shows the growth response of F.B. yeast to various levels of yeast extract under test conditions.

A large number of known compounds including the B vitamins, most of the familiar

amino acids, various saturated, unsaturated, and hydroxy acids, certain purines and pyrimidines, adenosine, adenylic acid, glutathione, sodium thioglycolate, and ascorbic acid were found to be inactive. Only cysteine and methionine showed any activity and neither of these produced an effect comparable with that of yeast extract.

None of the various source materials tested was as active as yeast extract. A commercial hydrolysate of casein (casamino acids) was found to resemble methionine in effect, but by measuring the growth stimulation of mixtures of this preparation and yeast extract and of methionine and yeast extract it was established that the activity of the hydrolysate did not depend solely on its content of methionine.

Substitution of pantothenic acid for β -alanine in the test medium resulted in an enhanced growth stimulation by casamino acids. A concentration of 10 γ per tube of the vitamin was as effective as 100 γ and was considerably more effective than 100 γ of β -alanine. If only 2 factors, pantothenic acid (present in yeast extract in nonlimiting concentration) and some other substance (contained in both yeast extract and casamino acids), were involved in the reversal of cyanide inhibition of growth, the growth stimulation produced by adding yeast extract to casamino acids in a pantothenic acid medium should represent an additive effect.

TABLE I
Growth Response of F.B. Yeast to Yeast Extract in Cyanide Medium.

Yeast extract added per tube, mg	Galvanometer reading
0	5
0.2	11
0.5	22.5
1.0	32
5.0	62
10.0	81

* Present address: Foods and Nutrition Department, Iowa State College, Ames, Iowa.

¹ Eppright, M. A., and Williams, R. J., *J. Gen. Physiol.*, in press.

² Pett, L. B., *Biochem. J.*, 1936, 30, 1438.

³ Stier, J. B., and Castor, J. G. B., *J. Gen. Physiol.*, 1941, 25, 229.

⁴ Williams, R. J., *Univ. Texas Pub.*, 1942, 4237, 7.

TABLE II.
Mannitol Recoveries by the Modified Method.

Substance to which mannitol and PAH were added	PAH mg % by weight	Mannitol mg %		Error %
		by weight	by analysis	
Water	0	10.0	9.92	-1
"	8.72	8.35	8.28	-1
"	8.72	8.35	8.10	-3
"	10.46	10.02	10.06	+0.04
"	10.46	10.02	10.17	+2
Urine filtrate	10.46	10.02	9.92	-1
Plasma "	10.46	10.02	9.87	-1
"	77.0	96.6	99.6	+3
"	77.0	96.6	97.7	+1

Each recovery was run in duplicate and the analytic results are the averages. Samples with like concentrations represent separate dilutions in different volumetric flasks, but from the same stock solutions.

reported.

Unfortunately the oxidation of PAH is not sufficiently quantitative to enable accurate correction of mannitol figures on the basis of that reaction.

Acetylation of PAH was found to prevent its oxidation and it was possible to accomplish this without detectable acetylation of mannitol taking place.

Hydrolysis of acetylated PAH to free PAH occurs when it is boiled in the $\text{KIO}_4\text{-H}_2\text{SO}_4$ solution usually used, but this can be avoided by replacing the sulfuric acid with acetic acid in the periodate solution. The sulfate which persists from the $\text{CdSO}_4\text{-H}_2\text{SO}_4$ solution used for precipitation of proteins does not seem to affect results.

Method. A modification of the periodate-iodide-thiosulfate method¹ which we have found to be satisfactory is as follows: 1. Glucose is removed from plasma and urine samples by yeast fermentation and protein is precipitated with cadmium sulfate. 2. Duplicate 2 cc samples of filtrate are pipetted into pyrex ignition tubes. 3. Roughly 1 cc of acetic anhydride is added from a bacterial pipette and the tubes are shaken several times during the next 5 or 10 minutes. 4. Exactly 5 cc of KIO_4 -acetic acid solution (0.06 Gm

KIO_4 dissolved in 100 cc of 20% acetic acid) is pipetted into the tubes. The tubes are covered with glass tears and boiled in a water bath for 20 minutes. 5. After cooling, about 0.5 cc of a 10% KI solution is added and the liberated iodine titrated immediately with sodium thiosulfate, using a starch indicator.

Recoveries of mannitol determined by this method in the presence of PAH are shown in Table II.

Summary. 1. The periodate-iodide-thiosulfate technic for mannitol determinations requires revision when para-aminohippurate is present. For each mg % of free PAH present, the mannitol recovery will be falsely elevated by from 0.25 to 0.30 mg %. 2. A modification of the technic is presented by which mannitol can be determined in the presence of PAH.²

¹ A method for mannitol estimations has been advanced^{4,5} which measures quantitatively one of the products of the reaction between mannitol and periodate. The presence of PAH should make no difference in such a procedure if a sufficient excess of periodate is used.

⁴ Coreoran, A. C., and Page, I. H., *Fed. Proc.*, 1946, 5, 130.

⁵ Page, I. H., personal communication.

TABLE III.
Growth Promoting Effect of Various Materials in Cyanide Medium.

Material tested	Apparent potency
Yeast extract (standard of reference)	1.0
Filtrate preparation	0.18
Eluate preparation	0.07
Casamino acids	0.20
Yeast extract (1 mg) ÷ filtrate (1 mg equivalent)	2.1
Yeast extract (1 mg) ÷ eluate (1 mg equivalent)	1.0
Yeast extract (1 mg) ÷ casamino acids (1 mg)	2.0
Filtrate ÷ eluate (equivalent amts of each)	1.0
Filtrate (1 mg equivalent) ÷ casamino acids (1 mg)	0.40
Eluate (1 mg equivalent) ÷ casamino acids (1 mg)	1.1
Filtrate ÷ nicotinic acid (10 γ per tube)	0.27
Filtrate ÷ folic acid (20 γ per tube)	0.22
Filtrate ÷ nicotinic acid (10 γ) ÷ folic acid (20 γ)	0.26

acid other than the ones tested or that its effect is a composite one due to several amino acids.

Even though nicotinic acid (or its amide) and folic acid were found to be somewhat stimulatory, neither individually nor variously combined did they behave in the same manner as did the eluate principle.

By repeated adsorption and elution from charcoal, a preparation was obtained which was approximately 8 times as active as that resulting from the initial treatment of yeast extract. A 6-fold concentration of the eluate principle was achieved by refluxing yeast extract with 95% ethyl alcohol and treating the soluble fraction once with Darco G-60.

Evidence indicating that the activity of eluate preparations does not depend on a single substance was derived by subjecting the principle to electrolysis at high voltage in the electrical transport apparatus described

by Williams and Truesdail.² The active principle was not concentrated in a single cell but was distributed throughout the 4-cell system employed.

Summary. Yeast extract has been shown to reverse the inhibition of yeast growth caused by cyanide. No known compound was found which duplicated the action of yeast extract, although methionine and cysteine had some "anti-cyanide" activity. Excess pantothenic acid had a beneficial effect on growth under certain conditions.

It has been demonstrated that the action of yeast extract depends on at least 2 substances which can be separated by charcoal adsorption. Indirect evidence indicates that the activity of both filtrate and eluate principles is due to more than a single substance.

² Williams, R. J., and Truesdail, J. H., *J. Am. Chem. Soc.*, 1931, 53, 4171.

15722

Effects of Various Anesthetic Agents on the Blood Pressure of the White Rat.*

N. M. SULKIN AND K. R. BRIZZEE. (Introduced by Albert Kuntz.)

From the Department of Anatomy, St. Louis University School of Medicine, St. Louis.

In an investigation involving the role of the autonomic nerves in cardiovascular disorders it was found desirable to measure the

* This work was supported by a grant from the Life Insurance Medical Research Fund.

blood pressure repeatedly in rats without injury to the animals and to determine the effect of anesthetic agents on the blood pressure. A survey of the literature showed that the apparatus used was varied and that de-

TABLE II.
Anti-Cyanide Activity of Yeast Extract and Casamino Acids, Separately and Combined, in Media Containing β -Alanine or Pantothenic Acid.

Amt of yeast extract added, mg	Extent of growth in various media					
	β -alanine medium		β -alanine medium + casamino acids*		Pantothenic acid medium	
	Galvanometer readings	Apparent mg units	Galvanometer readings	Apparent mg units	Galvanometer readings	Apparent mg units
0	6	0	23	0.65	6	0
0.5	21	0.5	49	3.0	22	0.6
1.0	28	1.0	56	4.4	28	1.0
5.0	59	5.0	69	>5	59	5.0
					73	>5

* The concentration of casamino acids was 10 mg per tube.

† The response to yeast extract alone in the β -alanine medium was used as standard.

The results in Table II show this not to be the case. It appeared, therefore, that at least 2 factors other than pantothenic acid must be involved. For all subsequent tests pantothenic acid was substituted for β -alanine in the culture medium.

The growth-promoting effect of yeast extract was improved by including in the medium any one of several reducing agents (ascorbic acid, sodium thioglycolate, glutathione, sodium hydrosulfite or cysteine). The optimum growth stimulation occurred in the medium containing 20 mg per liter of cysteine. For all of the experiments which followed this observation, the cyanide medium was supplemented with cysteine.

Further investigation provided evidence confirming the supposition that at least 2 substances other than pantothenic acid were responsible for the growth-promoting activity of yeast extract in a cyanide medium. These factors could be separated by adsorption with Darco G-60. The active material in the unadsorbed fraction was designated as the filtrate principle while that in the adsorbed portion which was eluted with an aqueous mixture of alcohol (40%) and ammonia (10%) was termed the eluate principle.

Although attempts to identify the filtrate and eluate principles with known compounds were unsuccessful, some information concerning their properties was obtained. Both principles are soluble in 70% ethyl alcohol and are unaffected by autoclaving in the presence of normal solutions of acid and base or by exposure to light. The filtrate principle is partially inactivated by treatment with nitrous acid and completely inactivated by hydrogen peroxide while the eluate principle is virtually unaffected by both of these reagents.

The results in Table III show other differences between the filtrate and eluate principles. The similar behavior of the filtrate principle and casamino acids made it seem likely that the filtrate principle was an amino acid. However, of a number of amino acids tested, all were inactive except norleucine which was inhibitory. The possibilities remain that the filtrate principle is an amino

TABLE I.
Effects of Various Anesthetic Agents on Systolic Blood Pressure of the White Rat.

	Mean, mm Hg.	Stand. dev., mm Hg.	Range, mm Hg.
Unanesthetized	124.8	7.54	102-140
Nembutal	104.1	7.78	86-115
Amytal	103.5	6.01	94-112
Ether (shallow)	117.0	4.62	110-126
Ether (deep)	104.0	4.91	95-110
Urethane	78.4	3.75	72- 84
Morphine	63.9	1.31	62- 70

made on each animal. If there was marked variation in the readings they were discarded and others were taken at a later time. The anesthetics used were given in the following dosages: pentobarbital-sodium (nembutal), 4 mg/100 g; sodium isoamylethyl barbiturate (amytal), 4 mg/100 g; ethyl carbamate (urethane), 20 mg/100 g; and morphine sulphate, 10 mg/100 g. Ether was applied by sponge. The other agents were all administered by intraperitoneal injection. In order to test the apparatus for high blood pressure some rats were made hypertensive by a high tyrosine diet and others were injected with adrenalin. All the tabulations obtained from the observations were treated to a statistical analysis.

Results. The experimental data obtained are summarized in Table I. In the unanesthetized group only one rat had a blood pressure as low as 102 mm Hg and only one had a pressure as high as 140 mm Hg. The remaining animals were in a range between 113 and 132. Animals of 2 groups, one with an average weight of 140 g and the other with an average weight of 200 g showed no significant differences in their blood pressures.

Rats that had been placed on a 10% tyrosine diet for 2 weeks preceding their blood pressure determination showed a mean arterial systolic pressure of 168 mm Hg. Those injected with 0.5 cc of 1/1000 adrenalin showed a rise in the systolic pressure from the normal level to 180 mm Hg.

The pressure then increased at a slower rate until it reached a peak of 210 mm Hg after which it receded to a normal level.

When ether was used there was considerable variation in the blood pressure determinations in individual rats. There was a marked decrease in blood pressure during the application of the ether sponge or shortly after, a rise in pressure when the effects of the ether subsided. Experiments on several animals indicate that there is a direct correlation between the decrease in blood pressure and the depth of the anesthesia.

The markedly lowered pressures that were obtained with the use of morphine and urethane may be explained on the basis of the vasodilator effects of these agents. The barbiturates (nembutal and amytal), according to Roth,¹⁴ act as cardiac depressants in the white rat which may account, to some extent, for the decrease in blood pressure.

Summary. The method of Byrom and Wilson as modified for this study is well adapted for the determination of blood pressure in the rat. The mean arterial systolic pressure in the unanesthetized animal was found to be 124.8 mm Hg. The mean pressures obtained by the use of anesthetic agents were as follows: nembutal—104.1, amytal—103.5, ether (shallow)—117, ether (deep)—104, urethane—78.4, morphine—63.9 mm Hg.

¹⁴ Roth, G. B., *Arch. Intern. de Pharm. et de Therap.*, 1935, 51, 179.

terminations of blood pressure were in many cases inconsistent. In some instances no mention was made of the anesthesia used.

The observations of Page and Reed,¹ Williams, Harrison and Grollman,² Kempf and Page,³ and Medoff and Bongiovanni⁴ are somewhat in agreement. All of their blood pressure determinations fall within a range of 100 to 140 mm Hg. Duncan, Hyman and Chambers⁵ reported blood pressure determinations from 90 to 140 in unanesthetized rats and from 70 to 120 in anesthetized animals. The studies of Telford, Swegart and Schoern⁶ are in agreement with those of the above workers, but in addition they found, in animals one year older, a blood pressure range between 84 and 95. Proskauer, Neumann and Graef⁷ reported normal blood pressure readings of 60 to 95 mm Hg. Duncan *et al.*⁵ found a decrease in blood pressure within a range of 70 to 120 in anesthetized animals and Byrom and Wilson,⁸ using ether, obtained blood pressures from 72 to 132 mm Hg.

Corcoran and Page,⁹ studying the effects of pentobarbital-sodium on the blood pressure of dogs, reported an increase in blood pressure, whereas Mylon, Winternitz and de Suto-Nagy¹⁰ found that nembutal simulates a condition like shock causing a lowering in blood pressure. Grimson, Kernodle and Hill,¹¹

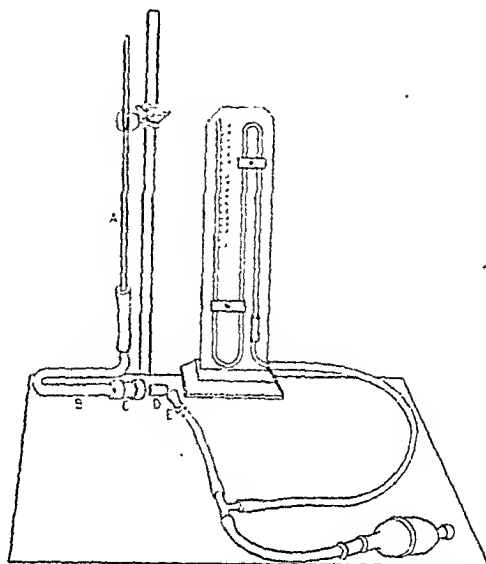


FIG. 1.

Blood pressure apparatus: A, 0.2 mm pipette; B, plethysmograph; C, gland; D, metal cuff; E, rubber pressure cuff.

studying the effects of various anesthetics on the blood pressure of dogs, obtained wholly inconsistent results.

In a study of spinal anesthesia in a large number of human cases Koster¹² found that it lowered the blood pressure which he believes due to a decrease in cardiac output.

Methods. In the present study, 40 female white rats of the Wistar Strain weighing 135 to 200 g were used. The room temperature at the time of the determinations was kept at $25 \pm 2^\circ\text{C}$. A modification of the tail plethysmograph method of Byrom and Wilson⁸ was used to determine blood pressure. It was found that the pressure cuff described by Griffith and Jeffers¹³ was most suitable. The plethysmograph consisted of a piece of bent glass tubing with a bore of 1 cm and a 0.2 mm bacteriological pipette (Fig. 1).

Unanesthetized rats were wrapped in a Turkish towel. In a short time they became quiescent and did not appear to be disturbed by the procedure. From 4 to 6 readings were

¹ Page, E. W., and Reed, R., *Am. J. Physiol.*, 1945, **143**, 122.

² Williams, J. R., Harrison, T. R., and Grollman, A., *J. Clin. Invest.*, 1939, **18**, 373.

³ Kempf, G. F., and Page, I. H., *J. Lab. and Clin. Med.*, 1942, **27**, 1192.

⁴ Medoff, H. S., and Bongiovanni, A. M., *Am. J. Physiol.*, 1945, **43**, 297.

⁵ Duncan, G. W., Hyman, C., and Chambers, E. L., *J. Lab. and Clin. Med.*, 1943, **28**, 886.

⁶ Telford, I. R., Swegart, J. E., and Schoern, C., *Am. J. Physiol.*, 1945, **143**, 214.

⁷ Proskauer, C. G., Neumann, C., and Graef, I., *Am. J. Physiol.*, 1945, **143**, 290.

⁸ Byrom, F. B., and Wilson, C. J., *J. Physiol.*, 1938, **93**, 301.

⁹ Corcoran, A. C., and Page, I. H., *Am. J. Physiol.*, 1943, **140**, 234.

¹⁰ Mylon, E., Winternitz, M. C., and de Suto-Nagy, G. J., *Am. J. Physiol.*, 1943, **139**, 313.

¹¹ Grimson, K. S., Kernodle, C. E., and Hill, H. C., *J. A. M. A.*, 1944, **120**, 218.

¹² Koster, H., *Arch. Surg.*, 1942, **45**, 596.

¹³ Griffith, J. A., and Jeffers, W. A., *The Rat in Laboratory Investigations*, 1942, J. B. Lippincott Co., Phila., p. 274.

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15723

A Fat-Soluble Material from Plasma Having the Biological Activities of Biotin.

WILLIAM TRAGER. (Introduced by Carl TenBroeck.)

From the Rockefeller Institute for Medical Research, Princeton, N.J.

It is now well established that biotin deficiency decreases the resistance of chickens and ducks to malaria parasites.^{1,2} It also appears to decrease the resistance of rats to *Trypanosoma lewisi*³ and of mice to mouse typhoid.⁴ A normal level of biotin must therefore be essential to the proper functioning of some general mechanism of resistance to infection. In the course of work directed toward discovering the nature of this resistance mechanism, it was found that the plasma of a variety of animals contains a previously undescribed material which, after hydrolysis by acid or enzymes, yields a fat-soluble substance having the biological activities of biotin but differing from it chem-

ically. This material is probably more intimately concerned in resistance to malaria parasites than is biotin itself. The changes in concentration which both it and biotin undergo in the plasma of chickens and ducks infected with malaria, and its effects on the multiplication of malaria parasites *in vitro* will be described elsewhere. It is the purpose of the present paper to present the data demonstrating the existence of the material and to relate some of its biological and chemical properties.

Of the various methods of microbiological assay for biotin, one of the most specific is that employing *Lactobacillus casei*.⁵ The *L. casei* assay has therefore been used almost exclusively. The medium and method of in-

¹ Trager, W., *J. Exp. Med.*, 1943, **77**, 557.

² Seeler, A. O., Ott, W. H., and Gundel, M. E., *Proc. Soc. Exp. Biol. and Med.*, 1944, **55**, 107.

³ Caldwell, F. E., and György, P., *Proc. Soc. Exp. Biol. and Med.*, 1943, **53**, 116.

⁴ Kligler, I. J., Guggenheim, K., and Herrnhaiser, H., *J. Infect. Dis.*, 1946, **78**, 60.

⁵ Shull, G. M., Hutchings, B. L., and Peterson, W. H., *J. Biol. Chem.*, 1942, **142**, 913.

⁶ Landy, M., and Dicken, D. M., *J. Lab. and Clin. Med.*, 1942, **27**, 1086.

⁷ Skeggs, H. R., and Wright, L. D., *J. Biol. Chem.*, 1944, **150**, 21.

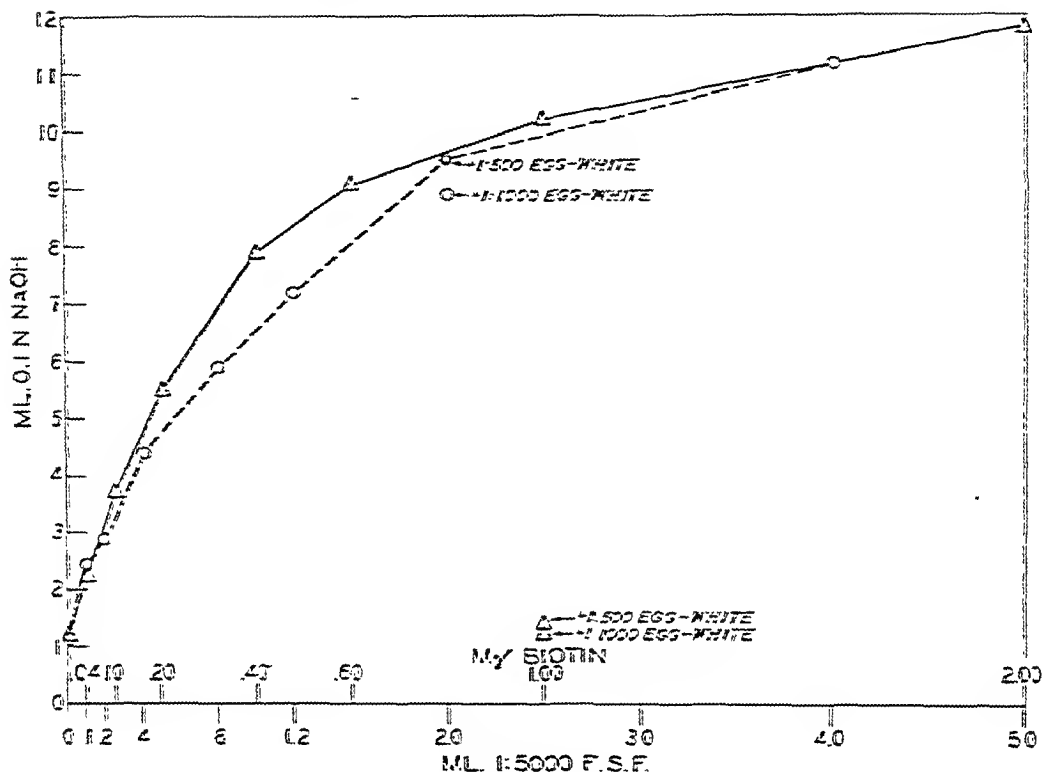


FIG. 1.

The response of *Lactobacillus casei* in an essentially biotin-free medium to the addition of different concentrations of: (1) Biotin (solid line and triangles). Amounts in $m\gamma$ per 10 ml culture tube given by upper numbers on abscissa; amounts in ml of the standard biotin solution by lower numbers; (2) FSE (dashed line and circles). Amounts in ml (ranging from 0.1 to 4.0) of a 1:5000 dilution of a known oil obtained from hydrolyzed horse plasma. The indicated triangles and circles show, respectively, the growth with 1 $m\gamma$ biotin plus 2 concentrations of fresh sterile egg-white, and the growth with 2 ml of the 1:5000 FSE plus 2 concentrations of egg-white.

filtered, 93 for the samples similarly treated but brought to pH 9, and 117 for samples which were filtered after being brought to pH 9. The recoveries after autoclaving in 6 N hydrochloric acid or 5 N sodium hydroxide were 79 and 62% respectively.

The biotin-active material which was removed by shaking hydrolyzed plasma with ether could be recovered in the ether extract. This fat-soluble material shall henceforth be designated as FSE. Of the various human plasma protein fractions,* fibrinogen, al-

bumin and γ -globulin contained very little FSE, while the fractions containing the α - and β -globulins and lipids were relatively rich in it. Fractions III-0 and IV-1, 1 W had the highest FSE content, with a biotin activity after acid hydrolysis of about 300 $m\gamma$ per g. A preparation of FSE from one of these fractions had a quantitatively similar effect on the growth of 5 species of lactic acid bacteria (*Lactobacillus casei*, *Leuconostoc mesenteroides* and *Streptococcus faecalis* R).

In order to study further the properties of FSE, 10 l of oxalated horse plasma were made 5 N with respect to sulfuric acid and autoclaved for one hour at 15 lbs. The mixture was brought to a pH of about 7.5 with 10 N sodium hydroxide and was filtered

* The plasma fractions were obtained through the kindness of Dr. L. C. Strong of the Harvard Medical School and were prepared under a recommendation by the Committee on Medical Research between Harvard University and the Office of Scientific Research and Development.

TABLE I.
Biotin Activity of Human and Duck Plasmas After Different Types of Treatment.

Plasma	Treatment	Activity as μ g biotin per ml plasma
Human	(1) Diluted in water.	2.0
	(2) Mixed with 10 X vol. of 3 N H_2SO_4 , autoclaved one hr, then neutralized.	11.4
	(3) Precipitated with trichloroacetic acid. Autoclaved 1 hr.	9.7
	(4) Precipitated with trichloroacetic acid. Precipitate removed by centrifugation. Supernatant autoclaved 1 hr.	1.8
Duck A	(1) Diluted in sterile water. Added aseptically to assay tubes.	3.2
	(2) Autoclaved 1 hr in 10 X vol. of 3 N H_2SO_4 . Neutralized.	14.6
	(3) Same as (2) but filtered before neutralization.	3.6
	(4) 24-hr treatment with takadiastase in acetate buffer, pH 4.7. Then neutralized and autoclaved.	15.7
Duck B	(1) Diluted in buffer, pH 8.0.	4.2
	(2) Same as (1) followed by ether extraction.	2.5
	(3) Autoclaved 1 hr in 10 X vol. of 3 N H_2SO_4 . Brought to pH 8.2.	10.0
	(4) Same as (3) followed by ether extraction.	1.3
	(5) 24-hr treatment with takadiastase in acetate buffer, pH 4.7. Then neutralized and autoclaved.	9.8
	(6) Same as (5) followed by ether extraction.	2.3

oculation were those described by Landy and Dicken⁶ except that the vitamins of the B complex were added in larger amounts,⁷ pure crystalline folic acid (obtained from Lederle Laboratories through the courtesy of Dr. Y. SubbaRow) was used at 25 γ per l of double-strength medium and pyridoxamine (Merck) was added at 10 γ per l. Growth was determined by titrating the acid produced with 0.1 N sodium hydroxide. Since it soon became apparent that a substance other than biotin was being measured in terms of its biotin activity, it was found advantageous to prolong the time of incubation of the assay tubes from the usual 3 days to 4. When this was done, the results obtained with 3 different concentrations of the same sample usually agreed within 10 to 15%.

Acid hydrolysis of plasma was accomplished by mixing 0.4 ml of plasma with 4 ml of 3 N sulfuric acid and autoclaving at 15 lbs for one hour, a procedure designed to liberate bound biotin.⁸ Plasma was also hydrolyzed by treatment with takadiastase,⁹ 5 mg of enzyme being used to 0.5 ml of

plasma diluted to 5 ml with acetate buffer of pH 4.7.

It was found that if normal plasma was hydrolyzed with sulfuric acid and filtered, either before or after neutralization, its biotin content was the same as that of plasma merely diluted in water, indicating that plasma does not contain any bound biotin. But if the hydrolyzed plasma was not filtered, a much higher biotin activity was found. Similar results were obtained after hydrolysis by takadiastase. All of the additional biotin activity resulting from the hydrolysis of plasma could be removed by shaking with ether, as well as by filtration. The shaking of unhydrolyzed plasma with ether removed a variable proportion of its already low biotin activity but did not affect the activity which appeared upon subsequent hydrolysis. Table I illustrates a few typical results out of many which have been obtained. In one series 6 μ g of pure biotin were added to duplicate 0.4 ml samples of plasma. These, and the corresponding samples without added biotin were submitted to various treatments and assayed for biotin. The % recovery of the added biotin was: 97 for the samples diluted in water, 89 for the samples autoclaved in sulfuric acid, brought to pH 7 and not

⁸ Lampen, J. O., Bahler, G. P., and Peterson, W. H., *J. Nutrition*, 1942, **23**, 11.

⁹ Luckey, T. D., Briggs, G. M., Jr., and Elvehjem, C. A., *J. Biol. Chem.*, 1944, **152**, 157.

Although the amounts and concentrations of FSF so far available have not permitted a complete test of its effectiveness against egg-white injury in animals, sufficient data have been obtained to show that it prevents the dermatitis produced in chickens by a diet high in egg-white. It also acts like biotin in preventing the increased susceptibility of chickens to infection with the malarial parasite *Plasmodium lophocera*, which otherwise occurs when the animals are maintained on an egg-white diet. The results of 2 experiments are shown in Table II. The diets used consisted of 80% of a chick-starting mash plus 20% of either powdered egg albumin or casein mixed with riboflavin at the rate of 5 mg per 100 g casein. In both experiments, FSF was administered in the breast muscle as the brown oil prepared from hydrolyzed horse plasma. The maximum amount of this which could be injected at one time was 0.2 ml, representing a biotin activity of 0.5 γ. In Exp. 1, the material was injected twice weekly and was reasonably well absorbed between injections. In Exp. 2, where 3 injections per week were given, pockets of the oil formed in the breast muscle. In both experiments the total dosage of FSF, in terms of microbiological biotin activity, was such that an equivalent amount of biotin also would not have given complete protection from biotin deficiency.¹⁰

All preparations of FSF so far examined have been found to be hemolytic for both duck and sheep red blood cells. The hemolysis is prevented by normal duck plasma. The biotin-like growth activity and the hemolytic activity have gone together through the following preliminary fractionation of the brown oil prepared from hydrolyzed horse plasma. A fraction which was difficultly soluble in alcohol but readily soluble in chloroform had little activity, as did a second fraction soluble in alcohol at room temperature but giving a copious white precipitate from cold alcohol. The active material was soluble in cold alcohol and was non-

separifiable. In the crude state it was soluble in acetone, but after its separation from the inactive separifiable fraction, it was insoluble in acetone. It is interesting that the addition of 5 parts of acetone to 1 part of an alcoholic solution of the non-separifiable fraction resulted in a quantitatively equivalent partition of both the growth and the hemolytic activities between the precipitate and the filtrate. Resaponification of the acetone insoluble material again yielded all the activity in the non-separifiable fraction. The minimum concentration of the various fractions which gave complete hemolysis was equivalent to a biotin activity of 0.2 to 0.5 my per ml, when 0.1 ml of 5% red cells was added to 0.9 ml of buffered mixture, incubated $\frac{1}{2}$ hour at 37°C and held overnight in a refrigerator. It is worthy of note that a crude preparation of FSF from a human plasma protein fraction gave complete hemolysis at a concentration with a biotin activity of 0.5 my per ml and slight hemolysis at a concentration of 0.15 my per ml.

It is apparent from the few properties of FSF thus far known that it cannot be biotin itself, which is not readily extracted in organic solvents¹¹ and which is inactivated by avidin.¹² FSF also does not correspond to any of the hitherto described analogues or vitamers of biotin, since all of these are either inactive in the growth of *L. rosei* and avert egg-white injury in animals or, if active, are like biotin itself inactivated by avidin.¹³⁻¹⁵

¹⁰ McBride, R. R., *Chickens and 2 Economic*, 1944, 2: 24.

¹¹ Eskin, R. E., McKinley, W. A., and Williams, R. J., *Science*, 1943, 92, 224.

¹² Ogilby, T. W., *Am. J. Med. Sci.*, 1942, 204, 570.

¹³ Bank, D., and Winkler, R. J., *Science*, 1943, 97, 57.

¹⁴ Drenth, R., and de Wijnbold, V., *Science*, 1944, 100, 129.

¹⁵ Schar, J. L., and Ganness, M., *J. Biol. Chem.*, 1945, 157, 151.

¹⁶ Eskin, R. J., Arnold, A. E., and Winkler, T., *Science*, 1945, 102, 35.

¹⁷ de Wijnbold, V., Drenth, R., Hofmann, K., and McBride, R. R., *Proc. Soc. Exp. Biol. and Med.*, 1942, 50, 374.

¹⁸ Eskin, R. J., *J. Biol. Chem.*, 1945, 157, 157.

¹⁹ Robinson, L. R., Hogan, A. G., and Miller, O. N., *Proc. Harvard Univ. Exp. Sta. Research Conf.*, 1944, 10: 17.

TABLE II.

Effect of Injections of FSF or Biotin in Chicks Fed a Diet High in Egg-White and Inoculated with *Plasmodium lophurae*.

In Experiment 1 the chicks were placed on the special diets at 7 days of age, the twice weekly injections were begun at 10 days and the inoculations were done at 25 days.

In Experiment 2 the chicks were placed on the special diet at 5 days, the injections (twice weekly for biotin, 3 times weekly for FSF) were begun at 8 days, and the inoculations were done at 20 days. In both experiments the injections were intramuscular.

Exp. No.	Diet	Injection	No. chicks	Avg degree of scabiness* of feet and mouth at days					Avg peak No. of parasites per 10,000 red cells	No. which died within 31 days
				19	25	27	31	35		
1	Egg white 20%	None	3	—	1.8	—	—	2.7	6780	0†
		Biotin 12 γ	4	—	0	—	—	0.1	2165	0
		FSF 1 γ	4	—	1.3	—	—	1.6	2505	0
	Casein 20%	biotin activity per week	4	—	0	—	—	0	2730	0
		None	4	—	0	—	—	0	2730	0
2	Egg white 20%	None	9	0.6	—	2.2	3.8	—	3340	5
		FSF 1.5 γ	8	0.2	—	1.0	1.0	—	3175	1
	" " "	biotin activity per week	8	0.1	—	0	0.2	—	1290	0
		Biotin 12 γ per week	8	0.1	—	0	0.2	—	1290	0

* An arbitrary scale ranging from 0 to 6 was used to express the extent of the lesions on the feet and at the corners of the mouth.

† Two of these chicks were very weak at 35 days, when they were killed. All the other chicks in this experiment were active at this time.

through hardened filter paper on a Buchner funnel. The filtrate contained very little activity and was discarded. The black residue was shaken with several large volumes of ether and the yellow ether extracts thus obtained were concentrated *in vacuo*. When all the ether had been removed, 31 ml of a brown oil were obtained. This was liquid at room temperature but solidified to a waxy material when refrigerated. The brown oil (prepared for assay as an opalescent emulsion made by diluting an aliquot with alcohol and then with water or buffer) had a biotin activity of 2.5 γ per ml, and represented a 70% recovery of the total activity of the hydrolyzed plasma. This material was used to demonstrate the ability of *L. casei* to dispense with biotin if adequate amounts of FSF are present. A dilute washed suspension of *L. casei* was inoculated into tubes of biotin assay medium free from added biotin but containing an adequate concentration of FSF. Twenty-four hours later, a loopful of the growth which had occurred

was inoculated to 2 similar tubes. Such sub-inoculation was repeated twice more. The 24-hour growth in the 4th transfer was washed and made into a dilute suspension in the usual manner.⁶ This was used to inoculate 2 series of tubes, one containing graded concentrations of biotin, the other graded concentrations of FSF. In each series there were included tubes with adequate biotin or FSF plus more than enough fresh sterile egg-white to inactivate the biotin activity. As is evident from Fig. 1, very similar curves were obtained relating the extent of growth to the relative concentrations of the 2 growth factors. However, the addition of egg-white completely inhibited growth in the tubes with biotin, but had no effect on growth in the presence of FSF. This result indicates that FSF is not merely a stimulatory substance, but can replace biotin in the growth of *L. casei*. All traces of biotin which may have been present in the culture medium must be considered to have been inactivated by the excess of fresh egg-white.

Newcastle Virus: Conversion of Spherical Forms to Filamentous Forms.

F. B. BANG. (Introduced by Carl TenBroeck.)

From the Rockefeller Institute for Medical Research, Princeton, N.J.

The filamentous forms of Newcastle virus seen in purified preparations¹ have not been seen in the crude allantoic fluid from infected embryos. This despite the fact that the virus is present in the allantoic fluid in the same concentration which in the purified material shows under the electron microscope great numbers of filamentous or tailed forms. Further work here reported indicates that the virus is roughly spherical in the allantoic fluid.

If allantoic fluid from infected chick embryos is centrifuged at 24,000 r.p.m. for 40 minutes and the pellet from this centrifugation is resuspended in water instead of saline, and this procedure repeated for another washing, the virus then shows a great predominance of roughly spherical forms under the electron microscope (Fig. 1b). This water suspension of virus has full activity (Table I) and remains stable for a number of days in the refrigerator (Table II). An occasional virus particle has a long thin attached tail, but gold shadowing fails to reveal other accessory structures. The virus particles are similar to those in the original allantoic fluid (Fig. 1a), with the exception that untreated preparations of infected allantoic fluid have never shown tailed forms.

If to this purified water suspension a little sodium chloride is added, a conversion from the spherical to tailed or filamentous forms occurs (Fig. 2). In the 3 experiments in which these results have been quantitated, the concentration of saline producing conversion has varied from .07 to .15 Molar. The conversion seems to be a progressive process and in individual experiments the extent of this conversion has varied with the salt concentration. Change of form, however, oc-

curs within a few minutes of adding the saline.

Filamentous forms seen in the saline suspensions may be reconverted to the spherical by transferring them back to a water suspension; this is done by centrifuging at 24,000 r.p.m. and resuspending in distilled water. These again will yield filamentous forms on the addition of saline.

The evidence for this conversion or change in form rests entirely on study of dried preparations with the electron microscope, and as yet we have no proof of the existence of these different forms in suspension.

That this conversion is not an artifact, however, is made likely by the following experiments. Dilute solutions (.02%) of formaldehyde progressively inactivate the virus over a period of days. If, an hour or two after formaldehyde has been added, .15 M saline is added, conversion readily takes place. If, however, formaldehyde has been allowed to act for 3 to 5 days before the addition of saline, the stimulus of the saline is no longer able to cause a change in form of most of the particles. Purified preparations in water inactivated by 10^{-3} M or 2×10^{-4} M mustard gas,^{2*} or partially inactivated by heating for 10 to 15 minutes at 50°C are also not converted to the filamentous form by the addition of saline. On the other hand, spherical forms which have remained in water without inactivation for as many as 7 or 8 days may be converted to the filamentous form by the addition of saline.

Summary. Study of Newcastle virns in allantoic fluid shows this virus to have a

² TenBroeck, C., and Herriott, R. M., *Proc. Soc. Exp. Biol. and Med.*, 1946, 62, 271.

* Dr. R. M. Herriott kindly treated a preparation with these concentrations of mustard gas.

¹ Bang, F. B., *Proc. Soc. Exp. Biol. and Med.*, 1946, 63, 5.

A number of growth-stimulating effects on *L. casei* by naturally occurring fat-soluble substances have been described.^{20,21} These effects have been shown to be due to certain fatty acids²²⁻²⁴ such as oleic acid, which had earlier been identified as a factor essential for the rapid growth of *Corynebacterium diphtheriae*.²⁵ Oleic acid and related compounds which stimulate the growth of *L. casei* in the presence of suboptimal concentrations of riboflavin or pantothenate do not permit growth in media lacking these vitamins.^{22,23} The situation with respect to oleic acid and biotin seems to be rather different.^{26,27} *L. casei* is evidently capable of continuous growth in a medium containing only traces of biotin but supplied with an adequate amount of oleic acid and adjusted to an initial pH of 5.6.²⁷ At first thought one might conclude that the activity of FSF is due to oleic acid, but a close inspection of the available facts makes such a conclusion untenable. Oleic acid in the absence of added biotin produced a maximal growth effect in a medium with an initial pH (before autoclaving)

of 5.6. If the pH was 6.5 or higher there was no growth. All the experiments with FSF were routinely done with a medium of pH 6.7-6.8 before autoclaving and 6.2-6.3 after autoclaving. Moreover, in a special experiment, the pH of the assay tubes was adjusted aseptically after autoclaving to 6.8-6.9. Fractions containing FSF had the same biotin activity under these conditions as at a pH of 6.2-6.3. FSF was fully active for *L. casei* even when all traces of biotin in the medium were rendered unavailable by an excess of avidin, a condition which has not been tested with oleic acid. FSF was also active against egg-white injury in chicks, an activity concerning which nothing has been reported for oleic acid. Finally the FSF activity was non-saponifiable and insoluble in acetone, and hence could not have been due to an ordinary fatty acid. It would seem likely, however, that there may be some relation between FSF and oleic acid, and that both are related to the utilization and function of biotin. A knowledge of the chemical nature of FSF may give a new insight into the mode of action of biotin.

Summary. The plasma of various species of animals yields, after hydrolysis with acids or enzymes, a fat-soluble material capable of replacing biotin in the growth of *Lactobacillus casei* and other lactic acid bacteria but not inactivated by avidin. When injected in chickens the material protected them from the injurious effects of a diet high in egg-white. Preparations containing the active material were found to be hemolytic, and in preliminary fractionations the growth and the hemolytic activities have gone together. The properties of the material do not correspond to those of oleic acid or of any previously described vitamers of biotin.

²⁰ Eckardt, R. E., Grörgy, P., and Johnson, L. V., *Proc. Soc. Exp. Biol. and Med.*, 1941, **40**, 405.

²¹ Feeney, R. E., and Strong, F. M., *J. Biol. Chem.*, 1942, **142**, 961.

²² Bauernfeind, J. C., Sotier, A. L., and Bowff, C. S., *Ind. and Eng. Chem., Anal. Ed.*, 1942, **14**, 666.

²³ Strong, F. M., and Carpenter, L. E., *Ind. and Eng. Chem., Anal. Ed.*, 1942, **14**, 909.

²⁴ Kodicek, E., and Worden, A. N., *Biochem. J.*, 1945, **39**, 78.

²⁵ Cohen, S., Snyder, J. C., and Mueller, J. H., *J. Bact.*, 1941, **41**, 581.

²⁶ Williams, V. R., and Fieger, E. A., *Ind. and Eng. Chem., Anal. Ed.*, 1945, **17**, 127.

²⁷ Williams, V. R., and Fieger, E. A., *J. Biol. Chem.*, 1946, **166**, 335.

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If allantoic fluid from infected chick embryos is centrifuged at 24,000 r.p.m. for 40 minutes and the pellet from this centrifugation is resuspended in water instead of saline, and this procedure repeated for another washing, the virus then shows a great predominance of roughly spherical forms under the electron microscope (Fig. 1b). This water suspension of virus has full activity (Table I) and remains stable for a number of days in the refrigerator (Table II). An occasional virus particle has a long thin attached tail, but gold shadowing fails to reveal other accessory structures. The virus particles are similar to those in the original allantoic fluid (Fig. 1a), with the exception that untreated preparations of infected allantoic fluid have never shown tailed forms.

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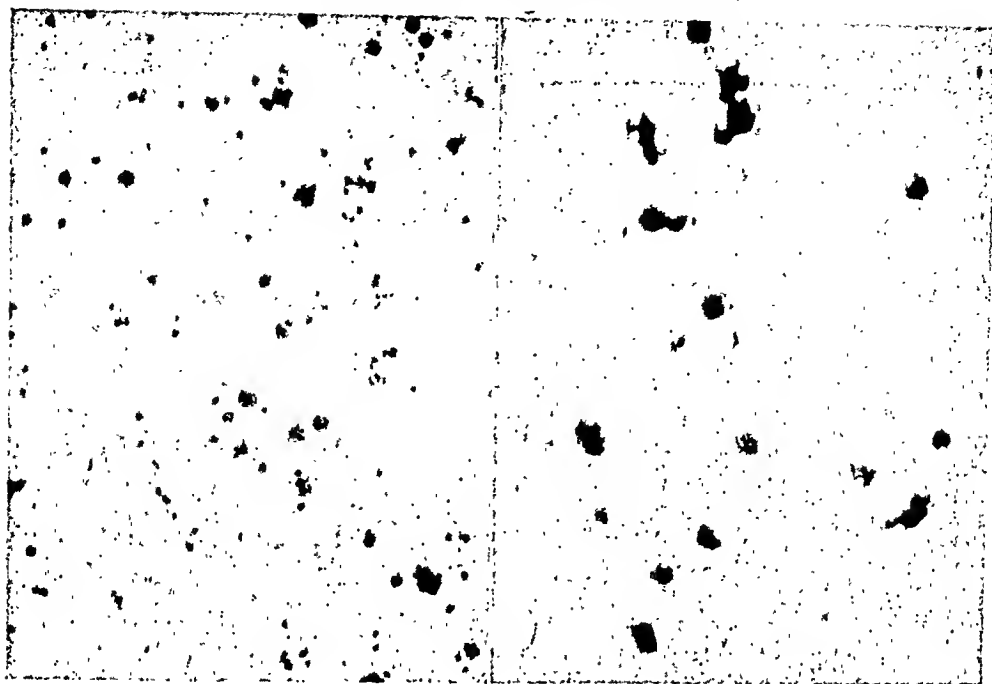


FIG. 1a. Electronmicrograph of unpurified Newcastle virus in allantoic fluid. $\times 17,200$.

FIG. 1b. Electronmicrograph of Newcastle virus resuspended in distilled water after purification by two cycles of differential centrifugation. $\times 17,200$.

TABLE I.
Purification of Newcastle Virus and Resuspension in Water.
Titer: 50% mortality of 10-day embryos.*

Exp. No.	Original allantoic fluid	Virys resuspended in		
		Water	.15 M NaCl	.15 M sucrose
1	10-8.3	10-8.7	10-8.5	
2	10-9.3	10-8.8		10-9.3

* All titrations are calculated on the basis of resuspension in a volume comparable to that of the original allantoic fluid.

TABLE II.
Stability of Newcastle Virus in Water and Saline at 4°C.
Titer: 50% mortality of 10-day embryos.

Time, day	Water	NaCl			Water (2nd exper.)
		.05 M	.15 M	.3 M	
1	10-9.2	10-8.6	10-9.0	10-7.2	10-8.7
4		10-8.7	10-8.2	10-6.0	
5					10-8.3
8	10-8.7				

roughly spherical shape when examined under the electron microscope. Most of the particles maintain this form when purified by

ultra-centrifugation and by transfer to water. The addition of .07 to .15 M sodium chloride to the solution converts it from the spherical

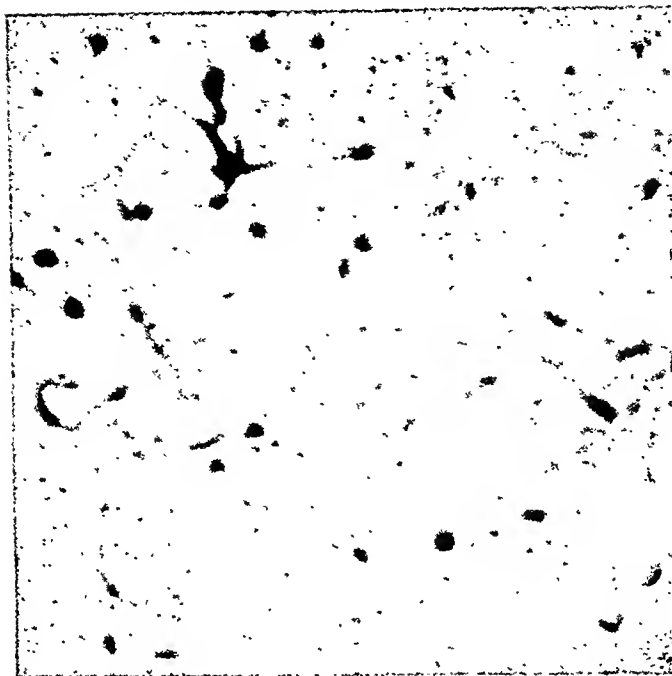


FIG. 2.
Electronmicrograph of same preparation as 1b in .15 M NaCl.
× 17,200.

to the filamentous form previously described. This conversion is prevented by partial in- activation with formaldehyde, mustard gas, or gentle heating.

15725

Effect of Hexaethyl Tetraphosphate on Choline Esterase *in vitro* and *in vivo*.*

KENNETH P. DuBOIS AND GEORGE H. MANGUN. (Introduced by E. M. K. Geiling.)

From the University of Chicago Toxicity Laboratory.

Hexaethyl tetraphosphate, $C_{12}H_{30}P_4O_{13}$, (HTP), has recently been introduced in this country as an insecticide. It was first used in this capacity by the Germans and was uncovered by technical teams following the close of the European phase of the war. The German scientists who were interrogated stated that it had a nicotine-like action and was used for the control of aphids as a sub-

stitute for nicotine. The authors of the present report have been unable to find any reference to its mechanism of action other than its nicotinic effects.

The present study was initiated following observation during routine testing¹ that animals showed symptoms similar to those pro-

¹ Botkin, A. L., Lipton, M. A., and Mangun, G. H., unpublished data.

² Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1936, 114, 495.

* Carried out under a contract with Medical Division, Chemical Corps, U. S. Army.

duced by diisopropyl fluorophosphate. Muscular twitching, tonic and tonic-clonic convulsions, involuntary defecation, micturition and salivation were observed. The parallelism was further confirmed by the production of miosis by instillation of a dilute solution in the eyes of rabbits. Maximal miosis occurred in about 5 minutes with one drop of solution containing 4 mg/ml of HTP. Pupillary diameter returned to normal in 5-12 hours in contrast to a parallel test with a solution of diisopropyl fluorophosphate (2 mg/ml) in which the miotic effect persisted for 3 days.

Experimental. Cholinesterase was measured manometrically employing a test system containing 0.3 ml 0.1 M acetyl choline bromide in a final volume of 3.0 ml of calcium-free Ringer-bicarbonate buffer (0.025 M NaHCO_3 , 0.15 M NaCl , and 0.04 M MgCl_2). This system contained either 50 mg homogenized brain, 100 mg homogenized submaxillary glands, 100 mg washed diluted red cells, or 100 mg serum. For ascertaining the effect of hexaethyl tetraphosphate (HTP) on insect cholinesterase the entire thorax from cockroaches was employed after removal of the chitin and homogenization. After gassing for 5 minutes with 95% nitrogen-5% carbon dioxide and equilibrating for 10 minutes at 38°C the acetyl choline was tipped from the side-arm into the main compartment of the Warburg vessel and readings were taken at 5-minute intervals for 30 minutes.

The effect of hexaethyl tetraphosphate on the cholinesterase of rat tissues and cockroach tissue *in vitro* was measured by the addition of solutions of the inhibitor dissolved in the buffer to the test system. The inhibitor was added immediately after solutions were prepared and incubated with the tissue throughout the gassing and equilibration period (15 minutes) before the addition of acetyl choline. A final concentration of 1×10^{-7} M hexaethyl tetraphosphate produced the following per cent inhibition of cholinesterase: brain 47, submaxillary 53, serum 60, erythrocytes 45, and cockroach tissue 58. Thus, hexaethyl tetraphosphate was an effective inhibitor of cholinesterase

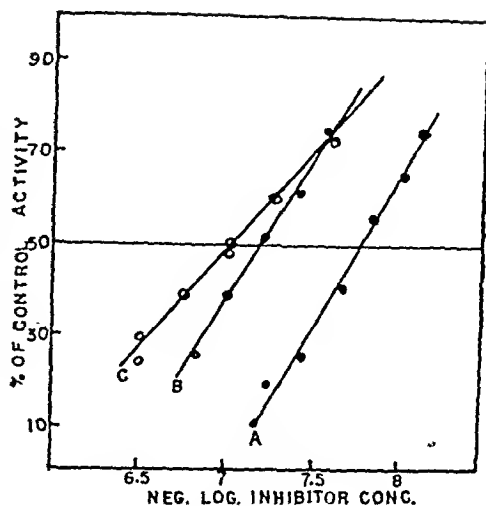


FIG. 1.

The effect of hexaethyl tetraphosphate, diisopropyl fluorophosphate, and a carbamic acid ester on rat brain cholinesterase *in vitro*. Curve A, hexaethyl tetraphosphate; Curve B, diisopropyl fluorophosphate; Curve C, carbamic acid, *N,N*-dimethyl-4-dimethylamino-5-isopropyl-phenyl ester, methiodide.

in vitro, all of the tissues showing similar sensitivity toward the compound.

Further evidence of the strong inhibitory action of hexaethyl tetraphosphate on cholinesterase was obtained by comparing its effect on the cholinesterase activity of rat brain *in vitro* with that of diisopropyl fluorophosphate (DFP) and carbamic acid, *N,N*-dimethyl-4-dimethylamino-5-isopropyl-phenyl ester, methiodide, 2 effective cholinesterase inhibitors. For this comparison a sample of hexaethyl tetraphosphate (Monsanto) and the other compounds were dissolved in the buffer and the measurement carried out as described above. The results shown in Fig. 1 indicate that 50% inhibition of brain cholinesterase activity was obtained by a final concentration of 1.6×10^{-8} M hexaethyl tetraphosphate, 6.3×10^{-8} M diisopropyl fluorophosphate, and 1×10^{-7} M carbamic acid ester. Thus, under the conditions of these experiments hexaethyl tetraphosphate was the most effective inhibitor and the similarity in the slopes of the curves in Fig. 1 indicate a similar type of inhibition by DFP and HTP.

Inhibition of cholinesterase *in vivo* was

TABLE I.
Effect of Hexaethyl Tetraphosphate on Cholinesterase Activity of Rat Tissues *in Vivo*.

Dose, mg/kg	% inhibition of activity			Toxicity
	Brain	Submaxillary	Serum	
1	4.5	22	100	0/3 died in 20 minutes
2	45.8	56.5	100	1/3 " " 20 "
3	75.8	83	100	2/3 " " 20 "
5	100	100	100	3/3 " " 5-6 "
10	100	100	100	" " 5 "

demonstrated by administering HTP intraperitoneally to rats and then measuring the cholinesterase activity of the brain, submaxillary glands, and serum by the *in vitro* test system previously described.

The results of these measurements are given in Table I. Values at 1, 2, 3, and 5 mg per kg are the averages of determinations on 3 animals and the 10 mg per kg represents one animal. The cholinesterase values for normal tissues from 10 animals expressed as microliters CO₂ per 50 mg fresh tissue per 10 minutes were: brain 102, submaxillary glands 28, serum 10, and red blood cells 7 and the per cent inhibition in poisoned animals was calculated from these control values.

These *in vivo* experiments demonstrate that HTP inhibits the cholinesterase of all of the tissues examined. Most sensitive was the serum which was completely inhibited

by doses of the compound which produce muscular twitching but no lethal effects in the animals. The inhibition of brain and submaxillary gland cholinesterase more nearly paralleled the symptoms and lethal action of the drug with submaxillary gland esterase being inhibited to a somewhat greater extent than brain. Twenty-four hours after the injection of a sublethal dose (1 mg per kg) into rats 35% of the serum activity had returned, at 4 days 70% of the serum activity had returned, and at 8 days the activity had returned to normal.

Summary. Hexaethyl tetraphosphate exerts a strong inhibitory effect on mammalian and insect cholinesterase *in vitro* and *in vivo*. This finding, in conjunction with its gross effects on animals suggests that its physiological effects may be at least in part due to its inhibition of this enzyme.

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Antigenic Structure of *Pasteurella pestis* and the Isolation of a Crystalline Antigen.*

E. E. BAKER, H. SOMMER, L. E. FOSTER, E. MEYER, AND K. F. MEYER.

From the George Williams Hooper Foundation, University of California, San Francisco, Calif.

The present report is a summary of investigations initiated in 1942 to extend the knowledge of the antigenic structure of *P. pestis*, and to use this information to place

* The work described in this paper was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the University of California.

plague prophylaxis on a sounder basis. The detailed experimental data will be reported at a later date.

All the studies, unless otherwise stated, were performed with dried plague bacilli of the virulent "Yreka" strain. The bacilli were grown on agar for 3 days at 37°C and suspended in saline. This was precipitated by one to 2 volumes of acetone cooled to

—70°C and left overnight. Repeated washings with acetone and drying *in vacuo* produced a bacterial powder of high antigenicity and toxicity (LD₅₀ for 20 g mice: 20-40 µg).

Extraction of the acetone-dried plague bacilli with neutral salt solutions (0.85%, 2.5% sodium chloride solutions or 0.4% sodium acetate solution) yielded a water-soluble and a water-insoluble antigenic component. The water-soluble fraction is quite toxic for mice and rats (LD₅₀ for 20 g mice, 8-15 µg), and has a high immunogenic value for these species, but a low value for guinea pigs. The water-insoluble portion is non-toxic for mice, and produces a very poor immunogenic response in mice and rats. However, this fraction, when tested as an alum precipitate, has a high immunogenic value, equal to that of whole plague bacilli, for guinea pigs.

The *water-soluble fraction* has been shown to contain at least 3 antigenic components: (1) A carbohydrate protein-soluble at 0.25 saturation of ammonium sulfate at pH 7.0-7.5, and precipitable at 0.3 saturation (Fraction IA). (2) A carbohydrate-free protein soluble at 0.3 saturation of ammonium sulfate at pH 7.0-7.5, and which crystallizes in the form of fine needles when the concentration of ammonium sulfate is raised to 0.33 saturation (Fraction IB). (3) A toxic fraction soluble at 0.33 saturation of ammonium sulfate at pH 7.0-7.5 and almost completely precipitated at 0.55-0.67 saturation (Fraction II). Both Fractions IA and IB are electrophoretically homogenous at pH 8.7 and pH 5.5. They both have approximately the same mobility at both pH values. Fraction IA has the following analytical values: Nitrogen 15.07%, sulfur 0.58%, phosphorus 0.08%, a strongly positive Molisch test, and is highly viscous. Fraction IB has the following analytical values: Nitrogen 15.71%, sulfur 0.59%, phosphorus 0.04%, a negative Molisch test, and a low viscosity. Both fractions are free of agar as determined by serological tests with a sample of horse serum containing antibodies to agar.

Fractions IA and IB are similar immunogenically. They both produce potent

antisera in rabbits, and will absorb all of the antibody from sera prepared against either. Sera to these fractions agglutinate plague bacilli to at least the same titer as sera prepared against whole plague bacilli. In one experiment, a serum to Fraction IA had as high a protective value for mice as serum to whole bacilli but, in contrast, had a very low protective titer for rats when compared with serum to whole bacilli. Serum to either of these fractions is incapable of neutralizing plague toxin. Both fractions will induce immunity in mice. Neither fraction is of value in producing immunity in guinea pigs.

Fraction II has never been obtained in a state approaching chemical or immunological purity. All preparations have been contaminated with Fraction I (IA and IB) in sufficient quantity to produce antibodies to Fraction I in rabbits. The best preparations had a toxicity (LD₅₀) for 20 g mice of 0.6 µg. However, it has been possible to prepare toxins free of Fraction I by serological techniques. It was observed that plague bacilli grown at room temperature were fully toxic, but extracts of such bacilli contained very little Fraction I. It proved possible to remove the residual Fraction I by absorption with either Fraction IA or IB antisera. The resulting absorbed extract showed no decrease in toxicity, and produced antisera in rabbits capable of neutralizing plague toxin. However, this antiserum was devoid of protective value for mice, did not agglutinate antigenically complete plague bacilli to a significant titer, nor react with either Fraction IA or IB except to a very slight degree in sensitive ring tests. Its value in inducing immunity in mice and guinea pigs has not as yet been investigated.

From the *water-insoluble fraction* or "residue" only small amounts of protein can be extracted with mild alkalis. Anhydrous phenol liquefied with 10-15% acetone dissolves approximately 25% of the residue. Both fractions, the phenol-soluble as well as the insoluble one, contained the antigen which protects guinea pigs.

The 2 soluble atoxic antigens (Fractions IA and IB) undoubtedly represent the "en-

velope" antigen of Schütze¹ and others. They are probably present on the surface of the bacilli, because antisera prepared against these fractions will agglutinate the strains of plague bacilli thus far tested. Only one avirulent strain, TRU,¹ obtained from Dr. Harry Schütze, Lister Institute, London, England, is not agglutinated by Fraction I sera, although it is slightly agglutinated by sera prepared against whole plague bacilli. This strain appears to be devoid of these 2 fractions. The exact relationship between Fractions IA and IB is not clear. Serologically, they are almost identical. Thus far, the crystalline Fraction IB proved of slightly lower immunogenic value. It might be suggested that in the intact cell the normal antigen is the carbohydrate protein Fraction IA, and that the crystalline protein is an artifact formed by disaggregation of the molecule during the death of the cell and subsequent treatment. All attempts to alter Fraction IA short of denaturation have failed.

It may be that plague bacilli contain an enzyme capable of converting Fraction IA to IB. Serologic studies have shown that Fraction I (IA and IB) is formed by the virulent strains tested, and the avirulent strain 1122 which forms a stable suspension in saline; avirulent strains showing salt instability produce only traces. These antigens are formed in quantity at 37°C; extracts of cells grown at room temperature contain relatively small amounts.

In view of the observed differences in response of the various laboratory animals to the different antigens of plague bacilli, it is considered advisable at the present time to use vaccines containing all of the antigens of *P. pestis* for prophylaxis of plague in man, and for the production of antiplague serum for therapeutic use.

The authors wish to thank Dr. M. Heidelberger, College of Physicians and Surgeons, for the gift of a sample of anti-influenza horse serum containing antibodies to agar; Dr. A. Elik, The Rockefeller Institute for Medical Research, for the microanalysis; and Dr. T. Shedlovsky, The Rockefeller Institute, for the electrophoretic analyses.

¹ Schütze, H., *Brit. J. Exp. Path.*, 1932, 13, 284; 1939, 20, 235.

15727

Liberation of Histamine and Heparin by Peptone from the Isolated Dog's Liver.*

M. ROCHA E SILVA,[†] A. EVELYN SCROGGIE, E. FIDLAR, AND L. B. JAUQUES.[‡]

From the Department of Physiology, University of Toronto.

In previous reports¹⁻³ one of us has shown that several agents (antigen, *e.g.* horse serum

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[†] Head of the Department of Biochemistry and Pharmacodynamics, Instituto Biológico, Sao Paulo, Brazil, Fellow of the Instituto-Canada, Brazil.

[‡] Head of the Department of Physiology, University of Saskatchewan, Saskatoon, Canada.

¹ Rocha e Silva, M., and Grana, A., *Arch. Surg.*, 1946, 52, 713.

² Rocha e Silva, M., Porto, A., and Andrade, S. O., *Arch. Surg.*, 1946, 53, 199.

³ Rocha e Silva, M., and Teixeira, R. M., *Proc. Soc. Exp. Biol. and Med.*, 1946, 61, 376.

in the sensitized animal, or peptone or *Ascaris* extracts) which *in vivo* produce a shock-like syndrome in the dog associated with a discharge of histamine and heparin from the liver, are unable to liberate significant amounts of these substances when perfused through the isolated liver, using Tyrode's solution as a vehicle. When citrated blood or heparinized blood is used as the perfusion fluid, detectable quantities of histamine and sometimes of heparin are discharged, but the total amounts that can be found in the perfusates are far smaller than those which are discharged in experiments *in vivo*. These

results lead to the conclusion that whole blood is necessary to produce the discharge of histamine and heparin from the hepatic cells in the forms of shock mentioned. In the experiments to be reported here, it has been possible on occasion to obtain the liberation from the liver of amounts of histamine and heparin greater even than *in vivo* and hence it has been possible to suggest the nature of the factors necessary for the liberation of these substances from the liver.

Methods. Ten dogs were used in the present experiments, all of them anaesthetized with sodium pentobarbital ("Ibital," Ingram and Bell). The liver was prepared for perfusion as described previously.² To avoid the use of anticoagulants in the blood, glassware treated with the silicone (Dri-Film, No. 9987, General Electric Co.) was used. In vessels so coated, blood will remain unclotted for 2 or 3 hours and the platelets are preserved for at least 30 minutes.⁴ It was assumed that, by thus completely excluding anticoagulants, conditions would be obtained more closely resembling those *in vivo*. All glassware entering into contact with the blood was carefully coated with the silicone, as described elsewhere,⁴ and contact with rubber tubing was reduced to a minimum. The blood was collected from the carotid through cannulae treated with silicone. Care was exercised to minimize trauma to the artery. Only the first 400 ml of blood were used for the perfusion. As soon as the animal had been exsanguinated, a cannula was inserted into the portal vein and the liver was perfused with warm (38°C) Tyrode's solution. The entire left lobe of the liver was tied off and was excised. The remaining portion of the liver was isolated and was placed in a warm chamber. The perfusion with warm Tyrode's solution was continued for 5 to 10 minutes, until the perfusate was free of blood. Three g of peptone in 10 ml of saline were then added to 250 ml of blood and the mixture was pumped rapidly through the organ. The perfusate was collected in small portions of 20 ml each and was assayed

for histamine and heparin. The organ was perfused further with 200 ml of blood and finally was washed out by prolonged perfusion with Tyrode's solution.

For the estimation of the histamine content of the perfusates, a piece of guinea pig ileum was employed. The heparin content was determined by the protamine-titration method of Jaques and Waters.⁵ In the experiments in which Tyrode's solution was used as the vehicle, the presence of heparin was investigated by concentrating the perfusates and then testing for metachromasia with the dye, Azure A.⁶ By this method, heparin can be estimated quantitatively in concentrations as low as 0.02 mg/ml, while it can be detected qualitatively with concentrations as low as 0.0005 mg/ml. We are indebted to Mr. E. Napke for conducting this test. The figures for total histamine and heparin given in Table I cover the whole discharge, since the perfusates were collected until only traces of histamine were detected by the biological test. In some of the experiments, after the perfusion with blood, a piece of the liver was taken in a 3.8% solution of sodium citrate and smears were prepared according to a technic previously described,² to observe the degree of disintegration of the clumps of platelets found within the liver parenchyma after perfusion with peptone. The same technic applied to the normal liver shows only intact, isolated platelets. Red cells, leucocytes and platelets were counted in the perfusing blood in 6 of the experiments and the degree of agglutination is indicated as the percentage of aggregated platelets to the total count.

The peptone was a sample of proteose-peptone (Difco) in 30% solution. In previous experiments we used a peptone which contained histamine as an impurity and it was necessary to rely upon increases in the histamine content of the perfusing blood. In these experiments the histamine was removed from the peptone by treatment with permutit,

⁵ Jaques, L. B., and Waters, E. T., *J. Physiol.*, 1941, **99**, 454.

⁶ Jaques, L. B., Mitford, M. B., and Macdonald, A. G., to be published.

⁴ Jaques, L. B., Fidler, E., Feldsted, E. T., and Macdonald, A. G., *Can. Med. Assn. J.*, 1946, **55**, 26.

TABLE I.
Total Histamine and Heparin Released from Isolated Dog's Liver Following Addition of Peptone to the Perfusion Fluid.

Dog, No.	Wt, kg	Liver, g	Perfusion fluid	Histamine, μ g	Heparin,† mg
11	16.0	170	Tyrodé's solution	26.8	None
17	15.5	402	" "	38.0	Traces
"	"	"	Heparinized blood—45 min. contact (8 units/ml)	93.7	1.75
19	17.6	365	Defibrinated blood	1,162.0	Not est'd
18	12.5	365	Heparinized blood—22 min. contact (8 units/ml)	538.0	Doubtful
16	10.1	295	" " 15 " " 5 "	2,511.0	10.5
13	22.5	280	Silicone blood‡	89.0	3.65
15	8.3	140	" "	417.7	5.45
14	29.6	359	" "	607.7	8.15
12	16.2	—	" "	3,313.0	16.5
20*	23.0	470	" "	8,550.0	25.4

* The figures for histamine and heparin in Dog 20 would not cover the whole discharge since after stoppage of the drainage with Tyrodé the histamine was still high (2.8 μ g per ml) and the heparin was estimated only in the tubes containing enough blood to be used in the protamine assay (350 ml of perfusate).

† The ratio of heparin : protamine under the conditions of this experiment was found to be 1:2.

‡ Blood collected and preserved in silicone-coated vessels.

as described by Gotzl and Dragstedt,⁷ thus permitting a much more accurate estimation of the histamine eventually liberated in the perfusion experiments. When the solution was diluted 25 times, as in the perfusion experiments, and was tested upon the guinea pig gut, only traces of histamine were found. It should be noted that a larger quantity of peptone was used in the present experiments than in those referred to above. Although we formerly used 1 or 2 g of peptone for 500 or 600 ml of blood (diluted 20% with Tyrodé's solution), in the experiments presented in this paper we have used 3 g of peptone added to 250 ml of undiluted blood. This difference was partly due to the fact that the sample of peptone used appeared to be less active than previous samples. The trypsin used was a crystalline sample containing 50% of $MgSO_4$ and produced by the Lehn and Fink Corporation. The protamine was a sample of salmine hydrochloride supplied by the Connaught Laboratories, University of Toronto.

Results. The total quantity of histamine and heparin liberated from the isolated liver of the dog, following the addition of peptone to the perfusion fluid, is presented in Table I. In agreement with results reported previously, peptone is capable of liberating only

small amounts of histamine and traces of heparin when Tyrodé's solution is used as the perfusing fluid. It is possible that even this small amount of histamine is liberated because of the presence of traces of blood retained within the perfused organ, since after the injection of peptone some blood appeared in the perfusate. In one experiment, the effect of crystalline trypsin on the perfused liver was tested. After washing the liver of Dog 11 free from peptone and traces of histamine with Tyrodé's solution, 50 mg of crystalline trypsin was added to the Tyrodé's solution perfusing the liver; 132 μ g of histamine and 1.5 mg of heparin were then released by the liver.

In contrast to the results obtained on adding peptone to Tyrodé's solution, when whole blood (especially blood kept unclotted by the use of silicone instead of anticoagulants) was used as a vehicle, enormous amounts of heparin and histamine were liberated. As shown in Fig. 1, most of the histamine and heparin was released at the beginning of the perfusion with blood and peptone. In some of the experiments the amount of histamine in the perfusates went up as high as 40 or 50 μ g per ml, in contrast to the 1.5 μ g per ml that has so far been the maximal content of histamine estimated in the blood of intact animals submitted to peptone shock.³ The variability shown in Table I in

⁷ Gotzl, F. R., and Dragstedt, C. A., *J. Pharm. and Exp. Ther.*, 1941, 74, 33.

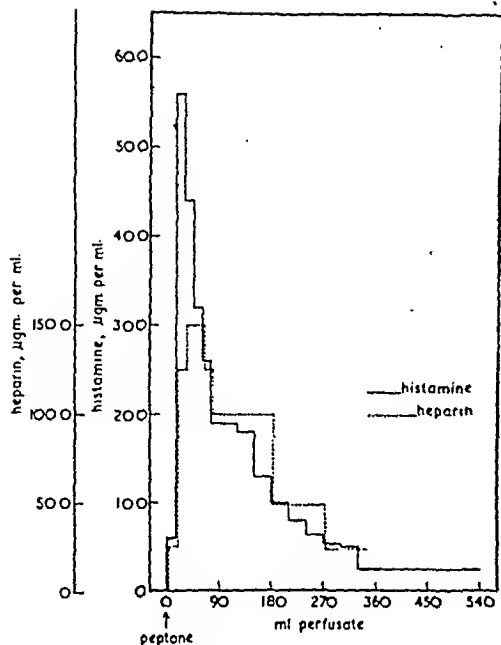


Fig. 1.

Dog No. 20. Liver perfusion with silicone blood + peptone. Discharge of histamine and heparin after passage of the blood through the liver. Note the sharp peak (56 μ g histamine per ml of perfusate) occurring before the first 50 ml of blood passed through.

the amounts of histamine and heparin released when "silicone blood" was used in the perfusion suggests that other factors (individual differences in the response of the liver from different dogs, the time of contact of the blood with peptone before coming in contact with the cells of the liver, etc.) are also operative in these experiments. These factors will be the subject of a separate investigation.

In the experiments presented in Table I even heparinized blood with peptone was able to liberate amounts of histamine and heparin far more conspicuous than those detected in previous experiments.³ This might be due to one or more of the following reasons: (1) the peptone was freed from histamine by treatment with permutil, thus permitting a much more accurate assay of histamine; (2) much higher doses of peptone were used than before; (3) the blood used was undiluted, while in the previous experiments the perfusion solution was blood which

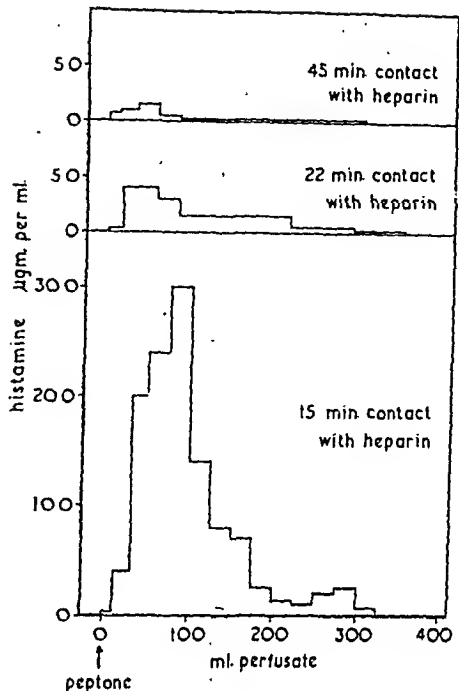


Fig. 2.

Three experiments with heparinized blood peptone. The maximal discharge of histamine was obtained when the blood was used 15 minutes after addition of heparin and the minimal when the blood was maintained for 45 minutes in contact with the heparin before passing through the liver.

contained approximately 20 to 25% Tyrode's solution. Whatever may be the reason for this discrepancy, heparin appeared to afford some protection against the action of peptone when enough time was allowed to elapse between the addition of heparin and the start of the perfusion experiment. As shown in Fig. 2, the maximal discharge of histamine was obtained when the blood remained in contact with heparin only 15 minutes. The minimal discharge resulted when the blood was kept for 45 minutes in contact with heparin. Due to the small number of experiments with heparin, further work is required to establish that heparin inhibits the discharge of histamine from dog's liver. Although defibrinated blood also permits the release of appreciable amounts of histamine and probably also of heparin (not estimated) by peptone, there is no question that the use of silicone-coated vessels for the collec-

TABLE II.
Leucocyte and Platelet Counts per mm³ of Perfusing Blood.*

Dog No.	Normal in silicone		After heparin		After peptone		Perfusate	
	Leucocytes × 100	Platelets × 1000	Leucocytes × 100	Platelets × 1000	Leucocytes × 100	Platelets × 1000	Leucocytes × 100	Platelets × 1000
15	62	411 (6%)†	Heparin not used		135	398	49	50 (87%)
16	77	415	79	368 (16%)	48	151 (67%)	41	70 (79%)
17	45	341	—	—	59	187 (91%)	5	12 (15%)
18	102	291	82	140 (42%)	94	227 (44%)	10	32 (25%)
19	25	363	79	46 (11%)	35	24 (62%)	14	14 (54%)
20	77	171	Heparin not used		74	129 (64%)	32	8.7 (48%)

* The red cells were used as an indicator of accuracy of sampling and of dilution, and in each experiment the leucocyte and platelet counts have been adjusted to the count of red cells in the normal blood.

† The figures in brackets indicate the degree of agglutination.

tion of blood, which thereby may approach its natural condition, permits the discharge of histamine and heparin in far more significant amounts than those obtained with defibrinated or heparinized blood.

Since the liberation of histamine and heparin from the liver by peptone appears to depend on the presence of whole blood, the question arises as to what constituents of the blood are involved in this action. It was previously found² with citrated blood as the perfusion fluid and antigen (horse serum) or *Ascaris* extracts as exciting agents that a sharp decrease in platelet and leucocyte counts occurred on passing the blood through the liver. As shown in Table II (Dogs 15, 17 and 20), this likewise occurred with the blood in silicone. Since the addition of peptone to the blood caused a marked agglutination of the platelets, the decrease in count may be attributed to trapping of these clumps in the hepatic capillaries. Confirming previous reports by Copley,^{8,9} heparin itself, when added to blood, caused considerable clumping of platelets and sometimes a sharp decrease in the count. The presence of heparin had little apparent effect on the action of peptone on the platelets and on the further decrease in the count when the blood passed through the liver.

Counts of leucocytes and of platelets cannot afford any direct evidence concerning the disintegration of these cells, since they might

be segregated in the network of capillaries and small vessels of the liver after forming clumps on the addition of peptone, as shown in Table II. Information regarding the destruction of these clumps was afforded by the observation of smears taken from pieces of the organ after the perfusion. In the experiments in which an excess of heparin had been used, the platelet clumps could be seen in great numbers throughout the slides, while in those experiments in which there was a considerable discharge of histamine and heparin the clumps were either absent or heavily damaged. The remains of platelets were seen but only very seldom were there any well stained clumps. This suggests that a disintegration of the platelets is associated with the liberation of histamine and heparin, and that while heparin will clump platelets, it will prevent their later disintegration.

The present report is intended to describe conditions under which it is possible to obtain regularly the liberation of histamine and heparin from the dog's liver with peptone. Using this technic we hope to elucidate the mechanism for this liberation. Rocha e Silva and Teixeira have postulated the activation of plasma trypsin as an intermediary in the liberation of histamine and heparin. The finding that the mechanism with peptone depends on the presence of blood, whereas crystalline trypsin by itself was observed to cause some release of histamine and heparin, is suggestive in this regard.

Conclusion. Experiments on the perfusion of dog's liver show that the presence of blood

⁸ Copley, A. L., *Am. J. Physiol.*, 1942, **133**, 248.

⁹ Copley, A. L., and Robb, T. P., *Am. J. Clin. Path.*, 1942, **12**, 416.

in the perfusing fluid is important for the production of an appreciable discharge of histamine and heparin by peptone. The best results were obtained when anticoagulants were excluded and the blood was preserved from clotting in silicone-treated receptacles. When Tyrode's solution was used as a vehicle for the perfusion, only small amounts of histamine and traces of heparin appeared in the perfusates. The experiments presented

in this paper do not afford any direct proof but are suggestive of the participation of platelets and possibly also of leucocytes in the mechanism of the discharge of histamine and heparin from the liver.

The authors are greatly indebted to Professor C. H. Best for facilities provided in the Department of Physiology, University of Toronto, and for his interest and support.

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Effect of Reticulo-Endothelial Blockade on Immunity to the Shwartzman Phenomenon.*

PAUL B. BEESON.

From the Department of Medicine, Emory University School of Medicine, and the Medical Service, Grady Hospital, Atlanta, Ga.

Experiments in this laboratory have shown that repeated intravenous injections of typhoid vaccine cause rabbits to develop a tolerance to the pyrogenic effect of the vaccine, and, furthermore, that this tolerance can be abolished by reticulo-endothelial (R-E) blockade.¹ Additional studies have shown that the development of tolerance to typhoid vaccine carries with it a similar tolerance to the pyrogenic effects of certain other Gram-negative bacilli, not serologically related to the typhoid bacillus.

In view of the fact that the pyrogenic activity of several species of Gram-negative bacilli has been shown to be the property of a carbohydrate component^{2,3} and that, at least in the case of *B. prodigiosus*, the same carbohydrate has been shown to be capable of inducing the Shwartzman phenomenon,⁴ it was thought that a study of immunity to

that form of injury might throw some light on the general problem of immunity to pyrogens. As a tool for study of this problem the Shwartzman phenomenon is advantageous because it provides an observable tissue injury in an area containing few R-E elements.

Materials and Methods. The source of bacterial toxin was the "Mitchell" strain of *E. typhosa*.¹ An "agar washings" filtrate was prepared by Shwartzman's method.^{5a} The potency of the filtrate was determined by titrating the minimum intravenous reacting dose against a constant skin preparatory dose.^{5b} Two different lots were prepared. Titration of the first showed 1 reactive unit in 1 ml of a 1:300 dilution, per kg of body weight; while in the second, 1 reactive unit was contained in 1 ml of a 1:400 dilution, per kg of body weight. The skin preparatory dose used in titrations and in all experiments was 0.25 ml of undiluted filtrate.

The rabbits were males, weighing 2 to 3

* Aided by a grant from the United States Public Health Service.

¹ Beeson, P. B., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61**, 248.

² Robinson, C. S., and Flusser, B. A., *J. Biol. Chem.*, 1944, **153**, 529.

³ Hartwell, J. L., Shear, M. J., and Adams, J. R., Jr., *J. Nat. Cancer Inst.*, 1943, **4**, 107.

⁴ Shwartzman, G., *Cancer Res.*, 1944, **4**, 191.

† Obtained from the Laboratories of the Georgia Department of Public Health.

⁵ Shwartzman, G., *Phenomenon of Local Tissue Reactivity and Its Immunological, Pathological, and Clinical Significance*, New York, Hoeber, 1937, (a) p. 35, (b) p. 26.

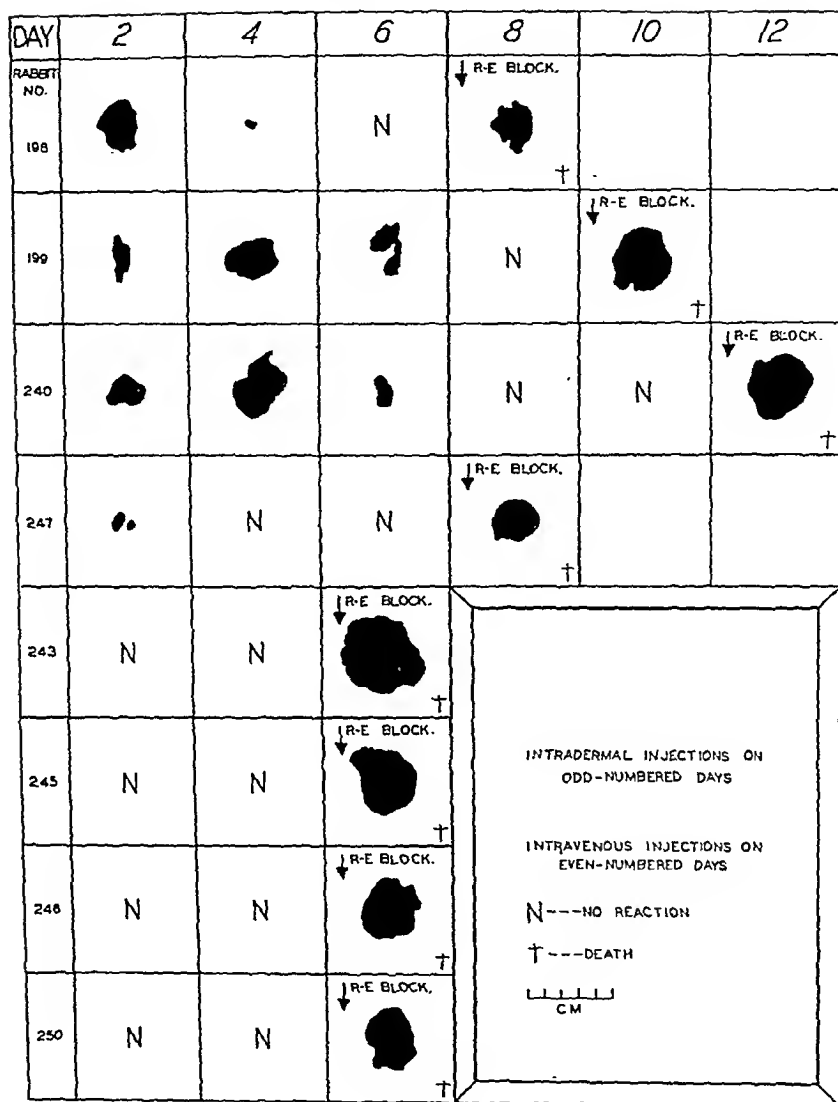


FIG. 1.

Graphic representation of the results obtained in 8 of the 20 rabbits given Thorotrast blockade. The upper 4 rabbits received the blockade after acquiring immunity, while the lower 4 were naturally immune. The shaded areas represent the area of hemorrhagic necrosis 5 hours after the intravenous injection of toxin. The scale in centimeters is indicated.

kg, either Chinchillas or New Zealand whites (Rockland). Tests were made on the skin of the abdomen. Since each animal was to have a series of tests, the abdomen was marked to designate 8 areas, and in the group of animals used in each experiment the individual tests were placed at different locations. The purpose of this was to lessen the

possibility of error due to injection in areas of low reactivity, since Schwartzman has found that there are differences in skin reactivity in different parts of the abdomen. Subsequent injections in each animal were made in adjacent areas, moving in a clockwise direction. The intravenous, or provocative dose, was 20 reacting units; this was admin-

istered 22 hours after the intradermal injection. The result was read 5 hours later. The only criterion used in determining the reaction was the presence or absence of a purplish hemorrhagic lesion. When positive reactions occurred the areas of skin hemorrhage were traced on transparent X-ray film, and then transcribed to permanent records.

For R-E blockade 2 agents were used: Thorotrast and trypan blue. Thorotrast is a commercial preparation (Heyden Co.) containing approximately 25% colloidal thorium dioxide. This was administered intravenously, in a dosage of 9 ml, 6 hours after the preparatory intradermal inoculation. Trypan blue was given in a 1% aqueous solution, the dose being 6 ml. This was given twice, 6 and 20 hours after the preparatory intradermal inoculation.

The plan followed in the experiments was to elicit the Shwartzman phenomenon in an animal repeatedly, the intradermal injection of one test being given on the day following the intravenous injection of the previous test. When an animal showed evidence of immunity by failing to react, the procedure was repeated with the addition of R-E blockade. In most of the experiments 2 negative results were obtained before the blockade, but in a few instances this was done after only one negative test.

Results. Of the 28 rabbits used in these experiments 13 were naturally immune; that is, no hemorrhagic reaction was visible 5 hours after the intravenous injection of 20 reacting units. The remaining 15 animals reacted positively 1 to 4 times before they too became immune.

Ten of the rabbits that were naturally immune received Thorotrast as a blocking agent, after 1 or 2 previous negative tests. In every case a positive reaction resulted. Ten rabbits that initially reacted positively but then showed negative reactions after 1 to 3 repetitions of the procedure also received R-E blockade with Thorotrast. Again, in every instance a positive reaction resulted. Fig. 1 shows a diagram of the results obtained with Thorotrast blockade in 8 of these animals, 4 naturally immune, and 4 with acquired immunity. Not only did R-E block-

ade cause the development of large areas of hemorrhagic necrosis at the sites of the intradermal inoculations, but also it caused death of all of these 8 animals.

When trypan blue was used as the blocking agent the results were not as uniform. In 3 naturally immune animals, blockade with this agent resulted in only one positive reaction. In 5 rabbits which had acquired immunity, blockade caused positive reactions twice.

The dose of bacterial toxin used in these experiments did not cause a single death in approximately 75 tests in this group of animals. Yet when R-E blockade was combined with the same dose of toxin, 17 of the 20 animals that received Thorotrast, and 3 of the 8 rabbits given trypan blue, died within 24 hours after injection of the intravenous dose of toxin. It is obvious then that R-E blockade not only alters the course of events at the site of the preparatory skin injection, but also that it markedly impairs the animal's general defense against the bacterial toxin.

The 3 animals which survived after exhibiting the Shwartzman phenomenon with Thorotrast blockade were retested immediately without blockade, and all 3 reacted negatively. Two of these, on receiving a second test combined with Thorotrast, again reacted positively and died within the succeeding 24 hours. The third survived a long sequence of tests, wherein it received a blocking dose of Thorotrast on 5 separate occasions. Following the last blockade it continued to react positively to a series of 10 tests, apparently being unable to regain a state of immunity. The reason for the unusual resistance of this animal, and for its eventual inability to become immune, is not clear.

Discussion. The results obtained with Thorotrast blockade in these experiments, and in our previously reported work on the febrile response to bacterial toxins¹ have been clear-cut and readily reproducible. This can be attributed partly to the fact that in both types of experiment the phenomenon being studied takes place within a few hours, allowing a test to be completed during a time

when functional impairment of the R-E system is at its height. Prolonged R-E blockade is almost impossible, because of the rapid recovery of function by the phagocytic cells and because of the fact that blockade is in itself a powerful stimulus to proliferation of these cells.⁶

Consideration of these results leads to the assumption that immunity to the Shwartzman reaction depends on ability of the R-E system to remove the bacterial toxin from the blood stream to such an extent that the tissues at the site of skin preparation are spared serious injury. R-E blockade permits the toxin to be delivered to the prepared skin area in a concentration sufficient to produce capillary damage and hemorrhagic necrosis. It also appears that other tissues suffer more extensive damage from the toxin in the presence of R-E blockade, as evidenced by the high fatality rate associated with blockade. Neither Thorotrast nor trypan blue would cause death in the doses used here.

These findings indicate that the functional state of the R-E system is of great importance in either natural or acquired immunity to the Shwartzman phenomenon. Shwartzman and others have provided a considerable body of evidence which indicates that specific antibodies may play an important part in acquired immunity. In view of the fact that phagocytosis by R-E cells is known to be enhanced in the presence of specific antibody, the 2 types of evidence do not necessarily conflict. Nevertheless, the evidence obtained in our other studies on immunity to bacterial pyrogens indicates that an acquired immunity can develop without the participation of specific humoral antibodies.

It is of interest that Thorotrast was much more effective in this action than was trypan blue. The dosages of both agents were comparable to those which have been used by other workers in studies of R-E blockade. One should note, however, that the actual quantity of colloidal thorium dioxide given

was approximately 20 times that of trypan blue.

Previous work on the effect of R-E blockade on the Shwartzman phenomenon has been somewhat contradictory. Gratia and Linz reported that blockade with large doses of India ink neither intensified nor lessened the local skin reaction.⁷ Two Italian reports, on the other hand, state that R-E blockade inhibits the Shwartzman phenomenon.^{8,9} In one of these studies trypan blue was used as the blocking agent, while in the other lithiocarmine was used. The findings reported here are in direct opposition to the conclusion that R-E blockade inhibits the Shwartzman phenomenon.

This work was undertaken principally in an attempt to elucidate the nature of the immunity developed by human beings and animals to the fever-producing effects of certain bacterial toxins. It would appear, however, that the findings may be of some use in other work involving the Shwartzman phenomenon, in that the variations in reactivity of different test animals can probably be lessened by the use of R-E blockade.

Summary. A study has been made of the effect of R-E blockade upon natural or induced immunity of rabbits to the Shwartzman phenomenon. Striking results were obtained when Thorotrast was used as the blocking agent. Rabbits that had shown a natural immunity, or had become immune following a series of previous Shwartzman reactions, responded, after the injection of Thorotrast, by developing typical areas of hemorrhagic necrosis. In addition to eliciting a positive skin reaction in a previously immune animal, R-E blockade increased the injurious effect of the bacterial toxin, causing death in the majority of animals tested.

Miss Elizabeth Roberts gave technical assistance in this work.

⁷ Gratia, A., and Linz, R., *Ann. Inst. Pasteur*, 1932, **49**, 131.

⁸ Ginnfre, T., *Pathologica*, 1937, **29**, 492.

⁹ Trizzino, E., and Caffarelli, F., *Riv. di Pat. Sper.*, 1939, **22-23**, 465.

⁶ Jaffe, R. H., *Physiol. Rev.*, 1931, **11**, 277.

Affinity of Avidin for Certain Analogs of Biotin.

LEMUEL D. WRIGHT, HELEN R. SKEGGS, AND EMLIN L. CRESSON.

From the Nutritional Laboratories, Department of Pharmacology, Medical Research Division, Sharp and Dohme, Inc., Glenolden, Penn.

Wright and Skeggs recently have described a procedure whereby the relative affinity of avidin for biotin and for biotin analogs possessing no microbiological activity readily may be compared.¹ The method was illustrated by a study of the relative affinity of avidin for the available spatial isomers of biotin.

This paper presents additional data on the avidin combinability of certain compounds having some structural similarity to biotin. The compounds studied (I-XIX) may be classified into 4 types: (1) acyclic analogs of desthiobiotin differing from desthiobiotin and its homologs by an opening of the imidazolidone ring (II-VII).^{2,*} (2) hydantoin derivatives differing from desthiobiotin homologs by the replacement of the methyl group with an oxygen atom (VIII,IX),* (3) "model" compounds (X-XIII) bearing some relationship to the original pyrimidine structure proposed and later retracted by Kögl *et al.* for α -biotin.^{3,4,*} (4) aromatic and cyclic aliphatic derivatives with a urea ring or a urea ring with an ω -carboxy aliphatic side chain (XIV-XIX).^{5,†}

The procedure employed¹ involved the ad-

dition of avidin to mixtures of biotin and the analog under investigation. The biotin and avidin were used in stoichiometric amounts and the amount of analog varied. In the case of compounds having definite avidin combinability an amount of biotin is available for the growth of *Lactobacillus arabinosus* that is equivalent to the amount of analog taken up by the avidin. The "relative affinity" is then expressed arbitrarily as the ratio of the concentration of analog to biotin at which one-half of the biotin remains free and available for growth of the test organism. It is obvious that the ratio will be low for analogs for which avidin has considerable affinity and high for those analogs that do not combine readily with avidin.

None of the compounds studied had any activity in lieu of biotin for promoting growth of *L. arabinosus*. Similarly, none of the compounds studied, in the amounts employed,

¹ Wright, L. D., and Skeggs, H. R., *Arch. Biochem.*, 1947, **12**, 27.

² Schultz, E. M., in press.

* We are indebted to Dr. E. M. Schultz of these laboratories for compounds II through XIII.

³ Kögl, F., Verbeek, J. H., Ersleben, H., and Borg, W. A. J., *Z. physiol. Chem.*, 1943, **279**, 121.

⁴ Kögl, F., and Borg, W. A. J., *Z. physiol. Chem.*, 1944, **281**, 65.

⁵ English, J. P., Clapp, R. C., Cole, Q. P., Halverstad, I. F., Lampen, J. O., and Roblin, R. O., Jr., *J. Am. Chem. Soc.*, 1945, **67**, 295.

† These compounds were supplied through the courtesy of Dr. J. O. Lampen of the Research Laboratories of the American Cyanamid Company.

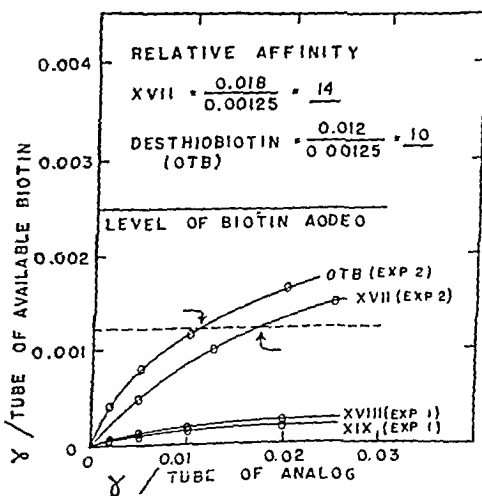


FIG. 1.

The relative affinity of avidin for biotin and biotin analogs.

TABLE I.
The Affinity of Avidin for Certain Analogs of Biotin.

Exp. No.	Biotin, γ	Analog, γ	Avidin, γ	Turbidity
1	0			84
	.00025			141
	.0005			176
	.00075			200
	.0010			220
	.0015			275
	.0025			318
	.0025		20	300
	.0025		50	250
	.0025		100	86
	.0025		200	83
	.0025	.002 XVIII	100	99
	.0025	.005 "	100	106
	.0025	.010 "	100	128
	.0025	.020 "	100	139
	.0025	.002 XIX	100	93
	.0025	.005 "	100	111
	.0025	.010 "	100	125
	.0025	.020 "	100	130
2	0			97
	.00025			163
	.0005			204
	.00075			246
	.0010			260
	.0015			300
	.0025			350
	.0025		20	335
	.0025		50	284
	.0025		100	122
	.0025		200	95
	.0025	.0050 XVII	100	212
	.0025	.0125 "	100	262
	.0025	.0250 "	100	300
	.0025	.050 "	100	330
	.0025	.002 DTB*	100	189
	.0025	.005 "	100	244
	.0025	.010 "	100	276
	.0025	.020 "	100	310

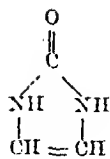
* *d,l*-Desthiobiotin.

had significant antibiotin activity for the assay organism.

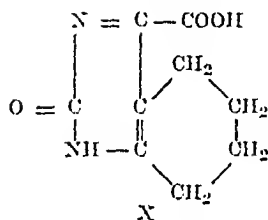
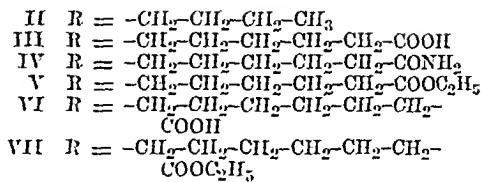
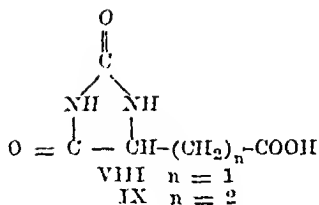
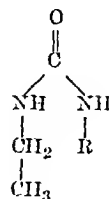
Combinability studies were carried out directly in the microbiologic assay medium by the aseptic addition of avidin just prior to seeding with *L. arabinosus*.

Of the compounds studied only No. XVII, XVIII, and XIX showed definite avidin combinability. Compound No. XVII, which differs from biotin in the replacement of the sulfur atom by a dimethylene group, had an affinity ratio of approximately 14. For reference *d,l*-epi-allobiotin has an affinity ratio

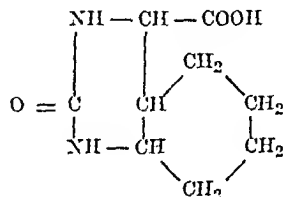
of about 3-6¹ while *d,l*-desthiobiotin has an affinity ratio of approximately 10 (Table I, Fig. 1). The specificity of the avidin combinability reaction further is illustrated by the fact that Compound XIX in which the ureylene group is attached to the cyclohexane ring at positions 3 and 4 rather than 2 and 3 with respect to the valeric acid side chain, although showing detectable avidin combinability (Table I, Fig. 1), had an affinity ratio too high for practical measurement. Compound XVIII in which the ureylene group is attached 3,4 to a cyclohexyl ring



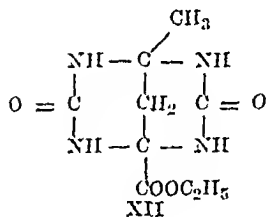
I



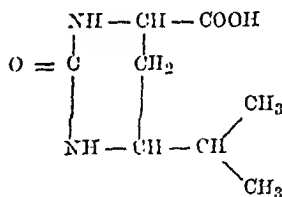
X



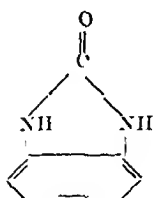
XI



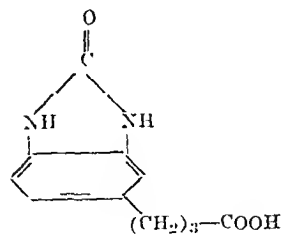
XII



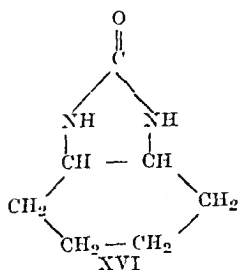
XIII



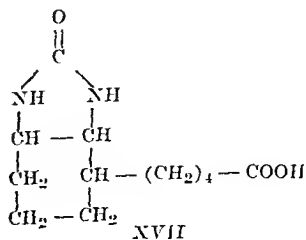
XIV



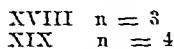
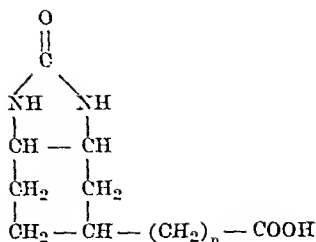
XV



XVI



XVII



containing a butyric acid side chain similarly showed definite avidin combinability but had an affinity ratio too high for practical measurement.

The failure of avidin to show significant affinity for the acyclic analogs of desthio-biotin (II-VII) is not unexpected. Opening of the imidazolidone ring would alter considerably the spatial arrangement of the molecule.

The inactivity of the hydantoin derivatives probably is attributable either to the shortness of the side chain in the compounds studied or to the existence of the ring predominantly in an enol form showing little resemblance to the cyclic urea ring of biotin.

While the resemblance of some of the pyrimidine derivatives studied to the original α -biotin formula of Kögl *et al.* is quite remote, Compound XIII is similar to the corresponding desthio derivative. The data do not permit a conclusion as to whether the failure of avidin to show significant affinity for this compound is due to the presence of a pyrimidine rather than an imidazolidone ring or to the absence of an ω -carboxy

aliphatic side chain of suitable length.

Winnick, Hofmann, Pilgrim and Axelrod⁶ have studied the inhibition of certain oxy-biotin derivatives with avidin. It was demonstrated that *dl*-oxybiotin, *dl*-oxybiotin methyl ester and hexahydro-2-oxo-1-furo-(3,4)-imidazole-4-pentanol (the alcohol corresponding to oxybiotin) combined with avidin. Approximately one unit of avidin was required to inactivate each compound for *Saccharomyces cerevisiae*. *Cis*-3,4-Diamino-2-tetrahydrofuranvaleric acid (oxybiotin diamino carboxylic acid) in which the cyclic urea ring is absent failed to combine with avidin. The procedures employed by Winnick *et al.* are capable of a qualitative interpretation but do not permit a conclusion as to the relative affinity of avidin for biotin in comparison with the affinity for the compounds studied.

Summary. The avidin combinability of a number of compounds having some structural similarity to biotin was investigated. The specificity of the avidin reaction was emphasized by the finding that of the 19 compounds studied avidin possessed significant affinity only for δ -(2,3-ureylene-cyclohexyl)-valeric acid (XVII), γ -(3,4-ureylene-cyclohexyl)-butyric acid (XVIII) and δ -(3,4-ureylene-cyclohexyl)-valeric acid (XIX). The relative affinity ratio for Compound XVII was found to be about 14. Compounds XVIII and XIX, although definitely capable of combining with avidin, had affinity ratios too high for practical measurement.

⁶ Winnick, T., Hofmann, K., Pilgrim, F. J., and Axelrod, A. E., *J. Biol. Chem.*, 1945, **161**, 405.

to 0.5 % of VDM (on the basis of nitrogen content) produced a 20- to 30-minute vaso-depressor effect in normal, 125 g rats, an effect similar to that produced by the injection of 0.5 cc of plasma from dogs in irreversible hemorrhagic or ischemic shock.

Four types of experimental animals were used:

(1) *Controls with liver and kidneys in the circulation.* Normal rabbits, and rabbits which had been partially exsanguinated according to a technique described by Engel,⁴ leaving intact the arterial blood supply to the liver via the hepatic artery. (2) *Arterial animals.* The renal artery and vein were tied off to exclude the kidneys from the circulation. (3) *Hepaticomized animals.* Complete exsanguination was carried out so as to exclude the liver from the circulation but leave the kidneys intact. (4) *Hepaticomized-arterial animals.* Complete exsanguination excluding both the liver and kidney from the circulation.

A series of 16 rabbits, anesthetized with Sarcocoll (25 mg per kg) were used in this group of experiments. Following the operative procedures the rabbits were transfused with 20 to 40 cc of whole blood or 5% albumin until the blood pressure became stabilized in the 90 to 100 mm Hg range. Control blood samples gave a neutral bio-assay. Two to 5 cc of the liver VDM concentrate were then injected intravenously and samples of femoral vein blood (2 to 5 cc) were removed within one to 2 minutes and at 10-minute intervals thereafter, until one hour had elapsed. For assay, about 0.5 cc of plasma was injected intravenously into normal rats in which the meso-appendix had been exposed for microscopic observation. The amount of VDM present in the injected blood samples was determined by noting the length of time that the terminal arterioles of the test rat remained unresponsive to epinephrine. The details of this method of assay are given in previous publications of Chambers and Zwahlen.^{1,2}

The disappearance of endogenous VDM

was studied in the partially exsanguinated rabbit preparation in which the hepatic artery was left as the sole source of blood supply to the liver. Temporary hepatic anoxia was produced by placing a clamp on the hepatic artery for varying periods of time, for the purpose of inducing VDM formation in the liver. The re-establishment of the hepatic circulation after a 45- to 90-minute period of liver anoxia led to the appearance of VDM in the blood stream. The disappearance of endogenous VDM was followed in 5 rabbits with either both the liver and kidneys in the circulation or with the kidneys excluded.

Results. A. The Disappearance of Exogenous VDM. 1. Controls, Liver and Kidneys in Circulation. The most rapid removal of VDM from the blood was found in the control series of animals in which both the liver and kidneys were intact. As shown in Fig. 1, blood taken one to 2 minutes after injection of vaso-depressor concentrate gave a 25- to 35-minute VDM bio-assay and then rapidly fell off in activity, becoming neutral within 30 to 40 minutes. The higher the initial blood concentration of VDM, the longer was the period required for the animal to clear the blood of this principle.

2. Arterial Animals. When the kidneys were tied off and the liver allowed to remain in the circulation, the VDM blood levels showed a more gradual decline, the depressor principle remaining in the blood somewhat longer than in control animals. In Fig. 2 it can be seen that the VDM was not completely cleared from the blood until 40 to 50 minutes had elapsed. The gradual disappearance of VDM in these animals closely resembled the progressive inactivation of VDM which occurred when liver slices were incubated aerobically *in vitro* with similar vaso-depressor solutions.²

3. Hepaticomized Animals. On the basis of *in vitro* inactivation experiments in which only the liver could inactivate VDM, it might be predicted that exclusion of the liver from the circulation should result in the accumulation of VDM in the blood. This, however, was not the case: in exsanguinated-hepaticomized animals with an intact kidney circula-

⁴Engel, F. C., Emerson, E. C., and Long, C. W. *Am. J. Exp. Med.* 1944, 79, A.

Hepato-Renal Factors in Circulatory Homeostasis.* II. Disappearance of Hepatic Vaso-Depressor Material Following Intravenous Administration.

S. BAEZ,[†] B. W. ZWEIFACH, A. MAZUR, AND EPHRAIM SHORR.
(With the technical assistance of S. Rosenfeld, D. Metz, and V. Bergman.)

From the Department of Medicine, Cornell University Medical College, and The New York Hospital, New York City.

Recent *in vivo* and *in vitro* studies^{1,2} have established the existence of a hitherto unrecognized vasodepressor principle which enters the blood stream in increasing amounts during the decompensatory stage of hemorrhagic and tourniquet shock. The vasodepressor principle, VDM, was found to be elaborated by the liver and skeletal muscle whenever the blood supply to those tissues was reduced below levels necessary to maintain an oxidative type of metabolism. The quantitative contribution of the liver is considerably greater than that of the skeletal muscle. Maximum blood levels are reached in experimental shock when the hypotension is profound and sufficiently prolonged to result in the development of a state unresponsive to transfusion, the so-called irreversible state.

Once the vasodepressor material appears during profound shock and precipitates the stage of vascular hypo-reactivity, it persists in the blood and is only temporarily diluted when the animal is transfused. The persistence of vasodepressor activity in the blood of shocked animals is in contrast to its transient effect when injected into the blood stream of normal rats for bio-assay. This points to the existence, under normal conditions, of mechanisms for removing this principle from the circulation. *In vitro* incubation experiments² have demonstrated that the healthy liver is the only tissue which

possesses an enzyme system capable of oxidatively destroying VDM. However, its removal from the blood stream in the living animal may be a more complex phenomenon, with several accessory mechanisms potentially participating in clearing the blood of VDM. Among these are the possible renal excretion of VDM into the urine, the inactivation of VDM by some component of the blood, and the counterbalancing of the vasodepressor effect by the release from the kidney of the oppositely acting vasoexcitor principle, VEM.

A consideration of the mechanisms concerned with the precise regulation of VDM blood levels should weigh the relative contributions of both the liver and kidneys in clearing VDM from the circulation. Experiments were therefore carried out to determine the rate at which endogenous or exogenous VDM was cleared from the blood stream of rabbits with the liver and kidneys intact, and with either the liver or the kidneys, or both, excluded from the circulation.

Methods. The disappearance of *exogenous* VDM was followed by the assay of blood samples taken at intervals following the intravenous injection of a VDM concentrate prepared from anaerobic beef liver (method of preparation used by Dr. A. Mazur to be published elsewhere). The vasodepressor activity of the heparinized plasma samples was measured by the rat meso-appendix technique³ which uses the reactivity of the terminal arterioles and precapillaries to epinephrine as an index of vasotropic effects. The potency of the liver concentrates was such the 0.1 γ

* Aided by grants from the Josiah Macy, Jr., Foundation and the Eli Lilly Company.

[†] Research Fellow from the Asuncion Medical School, Asuncion, Paraguay.

¹ Zweifach, B. W., Lee, R. E., Hyman, C., and Chambers, R., *Ann. Surg.*, 1944, **120**, 232.

² Shorr, Ephraim, Zweifach, B. W., and Furchgott, R. F., *Science*, 1945, **102**, 489.

³ Chambers, R., Zweifach, B. W., Lowenstein, B. E., and Lee, R. E., *Proc. Soc. Exp. Biol. and Med.*, 1944, **56**, 127.

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Four types of experimental animals were used:

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3. *Hepatectomized Animals.* On the basis of *in vitro* inactivation experiments in which only the liver could inactivate VDM, it might be predicted that exclusion of the liver from the circulation should result in the accumulation of VDM in the blood. This, however, was not the case; in eviscerated-hepatectomized animals with an intact kidney circula-

⁴ Engel, F. C., Harrison, H. C., and Long, C. N. H., *J. Exp. Med.*, 1944, 79, 9.

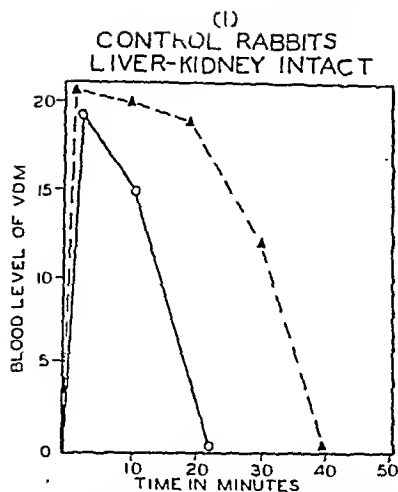


FIG. 1. *Control Rabbits*—includes a normal rabbit (O) and a partially eviscerated rabbit with the hepatic artery to the liver intact and with the kidneys in the circulation (Δ).

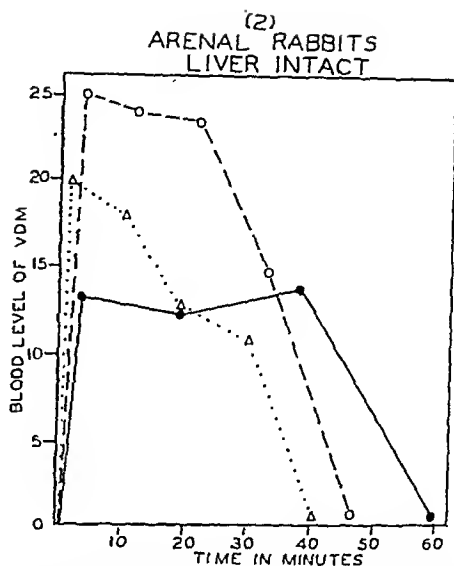


FIG. 2. *Arenal Rabbits*—includes 2 hepatic artery preparation animals (Δ, O) and one normal animal (●).

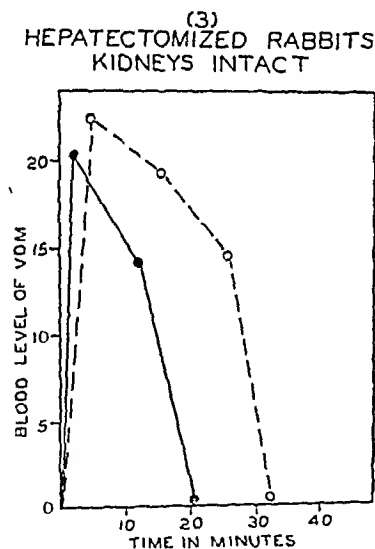


FIG. 3. *Hepatectomized Rabbits*—animals were eviscerated, leaving kidneys in circulation.

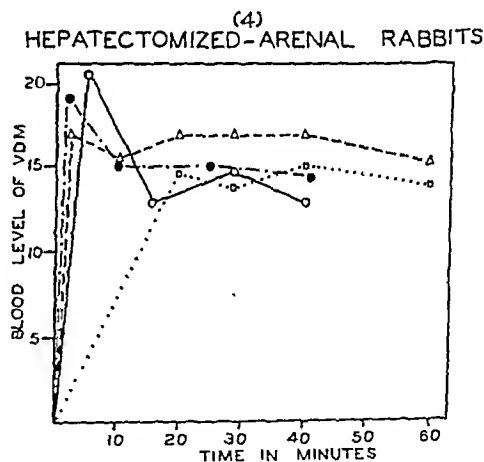


FIG. 4. *Hepatectomized-Arenal Rabbits*—animals were hepatectomized and renal pedicle tied off.

FIG. 1, 2, 3, 4.

Graphs of VDM blood levels following the intravenous injection of a vasodepressor concentrate prepared from anaerobic beef liver. The amounts of vasodepressor material in the blood were determined by the rat meso-appendix test and are expressed along the ordinate axis in terms of the number of minutes that the arterioles and precapillaries remained unresponsive to epinephrine.

tion, VDM disappeared from the blood within 30 to 40 minutes after its intravenous administration (Fig. 3). Since kidney tissue cannot inactivate VDM, another renal mechanism, presumably excretion, appeared to be the means for its removal under these con-

ditions. This inference was confirmed by experiments on the dog in which urine samples were collected and tested for vasodepressor activity. A dog was hepatectomized and the kidneys left in the circulation. Blood and urine samples were taken following the

intravenous injection of the liver vasodepressor concentrate, the urine being collected from the bladder by means of an in-dwelling catheter. In order to eliminate the undesirable effects on the rat test of the concentrations of urea and electrolytes in the urine, it was dialyzed against distilled water overnight in the refrigerator before being tested. Considerable quantities of VDM were found in the urine after the intravenous injection of VDM, whereas the control urine showed only a mild vasodepressor activity. The excretion into human urine of a principle having similar vascular depressor effects has recently been demonstrated in this laboratory by Dr. R. F. Furchgott.

The difference between the controls and the arenal animals points to the participation of the kidney in the removal of VDM from the blood. Additional evidence for such participation was obtained from experiments in which meso-appendix assays were made on both normal rats and arenal rats using the same VDM sample. When the kidneys were tied off, the depressor effects of VDM on the peripheral vessels persisted for longer periods than in normal rats.

Blalock⁵ and more recently Van Slyke and co-workers⁶ have shown that renal blood flow is reduced to negligible levels in profound shock. The loss of the renal-excretory route for VDM disposal undoubtedly serves as an additional factor to perpetuate the blood vasodepressor activity and thereby contributes to the development of an irreversible state.

4. *Hepatectomized-Arenal Animals.* The kidneys and the liver appear to be the only tissues concerned with the removal of VDM from the blood. When both these organs were excluded from the circulation of the rabbit, the blood level of VDM remained significantly unaffected over a period of an hour, the duration of the experiment (Fig. 4).

The persistence of VDM activity in the blood in the absence of the liver and kidney

indicated that the blood *per se* was not concerned with VDM inactivation. This was further confirmed by the observation that plasma or serum suffered little or no loss in VDM activity during aerobic incubation at 37.5°C for 2 to 3 hours.

Previous *in vivo* experiments² have demonstrated that the capacity of the liver to inactivate VDM is progressively impaired in shock by the hypoxic state in the organ. In the present series a similar situation was encountered in 3 rabbits which had developed profound hypotension for about 60 minutes following accidental blood-loss during the evisceration procedure. The rabbits were then transfused with large amounts of whole blood until they had returned to normal blood pressure levels. In one animal the kidneys were then tied off and the VDM concentrate injected into the blood stream. No significant removal of VDM occurred and the animal went into profound shock following the removal of only 8 cc of blood for testing purposes. In the other 2 rabbits the kidneys were left in the circulation. Despite the presence of the kidneys, the intravenous administration of VDM was likewise followed by its persistence in the circulation and by a progressive fall of blood pressure to shock levels.

B. *The Disappearance of Endogenous VDM.* These studies were made on a special preparation in which the gastro-intestinal tract was completely removed depriving the liver of its portal blood supply but leaving the hepatic artery intact. Long and co-workers⁶ have found that the liver in such animals is maintained in a healthy condition for at least 4 to 5 hours, the duration of their observation. In our own studies, the aerobic state of the liver metabolism under these circumstances is indicated by the absence of VDM in the blood. When the hepatic artery was occluded for 60 minutes and then released, considerable amounts of VDM appeared in the blood stream within 15 minutes. In animals with an intact renal circulation, the VDM disappeared from the blood within 60 minutes after release of the hepatic clamp. When the kidneys were tied

⁵ Blalock, A., and Levy, S. E., *Ann. J. Physiol.*, 1937, **118**, 734.

⁶ Van Slyke, D. D., and co-workers, *Conference on Hemorrhage*, Ann. N. Y. Acad. Sci., 1946.

off before releasing the clamp on the hepatic artery, large amounts of VDM accumulated in the blood. The blood pressure fell to shock levels within 45 minutes. Evidently, the VDM inactivating mechanism of the liver had been damaged by the period of anaerobiosis and was no longer capable of inactivating the VDM in the blood.

Summary. *In vitro* studies have shown that the vasodepressor principle, which appears during the hypo-reactive stage of shock and results from the anoxia of liver and skeletal muscles, can be inactivated under aerobic conditions by healthy liver slices. The present study was concerned with the mechanisms by which endogenous and exogenous VDM are removed from the circulation of the living animal. The exogenous VDM was concentrated and purified from saline extracts of anaerobic beef liver. The endogenous VDM was released into the blood following liver anoxia produced by tem-

porary occlusion of the hepatic artery in a partially eviscerated preparation. Two mechanisms for VDM removal were revealed: (1) its inactivation by the healthy liver; (2) its excretion into the urine by the normal kidney. A preliminary period of hepatic anoxia rendered the liver incapable of inactivating VDM *in vivo*, presumably through anoxic damage to the hepatic enzyme system for this function. This situation is analogous to the progressive impairment of the VDM inactivating mechanism in the liver which develops during the course of shock and which is considered responsible for the perpetuation of the hypo-reactive state of the peripheral vascular system. The loss of the renal excretory function for VDM during hypo-reactive shock deprives the animal of an important means of clearing the blood of VDM and thereby aiding in liberating the vascular bed from this decompensatory vasotropic principle.

15731

Functional Components of the Greater Superficial Petrosal Nerve.

JAMES O. FOLEY.

From the Department of Anatomy, Medical College of Alabama, Birmingham, Ala.

Larsell and Fenton¹ early emphasized the importance and pointed out the inadequacies of our knowledge of the greater superficial petrosal nerve. Later Chorobski and Penfield² added emphasis to the subject by demonstrating that the nerve is an important pathway for vasodilator fibers of the seventh cranial nerve to the pial arteries. More recently Foley and DuBois³ experimentally separated the functional components of the nerve in a limited number of animals and described its varieties of sensory and motor

fibers. Numerative studies on the functional components of the greater superficial petrosal nerve, in a satisfactory number of animals, are needed to complete our knowledge of the composition of the nerve.

Methods. The motor nerve fibers of the greater superficial petrosal nerve of the right side of 9 cats and one dog were eliminated by cutting the rootlets of the facial nerve within the cranium. This operation produced a nerve that, aside from an occasional easily identifiable³ sympathetic fascicle, consisted only of sensory axons derived from the geniculate ganglion of the seventh cranial nerve.

After allowing a minimum of 14 days for disappearance of the motor axons, the right degenerated and left normal nerves of each

¹ Larsell, O., and Fenton, R. A., *Laryngoscope*, 1928, **38**, 371.

² Chorobski, J., and Penfield, W., *Arch. Neur. and Psychiat.*, 1932, **28**, 1257.

³ Foley, J. O., and DuBois, F. S., *J. Comp. Neur.*, 1943, **79**, 79.

TABLE I. The Greater Superficial Petrosal Nerve.

Animal No.	Surgical procedure	Operated (right) side				Unoperated (left) side			
		Ratio* total motor axons	Myelinated axons	Ratio* myelinated motor axons	Unmye- linated axons	Ratio* unmye- linated motor axons	Total axons	Myelinated axons	Unmye- linated axons
Cat 126†	Intraaural sect. roots	2.07	7068-1137m	1.61	898-511m	5.77	2446	1843	603
Cat 2†	Intraaural sect. roots	2.21	4248-1491m	3.50	4218-381m	0.90	2717	1915	802
Cat 5†	Intraaural sect. roots	3.11	4738-1070m	2.26	188-457m	25.14	2018	1543	475
Cat 127†	Intraaural sect. roots	1.05	5808-415m	0.76	2208-395m	1.79	1640	1025	615
Cat 161†	Intraaural sect. roots	2.83	5168-1092m	1.96	928-719m	7.81	2329	1511	811
Cat 162†	Intraaural sect. roots	6.08	2528-1678m	6.65	1148-550m	4.81	2594	1930	664
Cat 163†	Intraaural sect. roots	2.70	5308-1100m	2.07	488-465m	9.68	2143	1630	513
Cat 164†	Intraaural sect. roots	4.79	2728-1709m	6.17	978-61m	0.62	2139	1981	158
Cat 170†	Intraaural sect. roots	2.27	4008-840m	2.10	1258-352m	2.81	1717	1240	477
Axon averages for all nerves of cat		2.67	4618-1164m	2.52	1368-432m	3.17	2193	1625	568
Dog 1†	Intraaural sect. roots	2.63	6648-1635m	2.45	2908-892m	3.01	3487	2299	1188

* The single number represents the ratio of motor axons as derived by dividing the number of motor fibers by the number of sensory fibers.
 † Axons right and left nerves stained with pyridine silver technic.
 ‡ Axons right and left nerves stained with Bodian technic.

animal were secured and processed for enumeration of the myelinated and unmyelinated nerve fibers with a variant of either the pyridine silver⁴ or Bodian technic.⁴

Complete counts were made in selected cross sections⁴ of the sensory axons which remained in the degenerated nerves, whereas, the combined sensory and motor nerve fibers of the nerves of the unoperated sides were estimated in transverse sections by means of the strip method.⁵ The number of motor axons that were present in the deafferented nerves before operation was approximated by subtracting the sensory fibers which remained in the degenerated nerve from the combined motor and sensory axons which were enumerated in the control nerve.

The total number of nerve fibers and the unmyelinated axons in both the degenerated and normal nerves was counted or estimated. The number of myelinated nerve fibers was then derived by subtracting the unmyelinated type of axons from the total number of nerve fibers.

Results. The normal greater superficial petrosal nerve of the cat (Table I, unoperated side) contains from 1640 (cat 127) to 2717 (cat 2) combined sensory and motor axons. The myelinated sensory and motor axons of the intact petrosal nerve vary from 1025 (cat 127) to 1981 (cat 164), whereas only 158 (cat 164) to 811 (cat 161) are without myelin sheaths. The single normal specimen of the petrosal nerve of the dog (dog 1, Table I) has 3487 axons which are divided into 2299 myelinated and 1188 unmyelinated nerve fibers.

Of the deafferented nerves of the cat which contain only sensory fibers (s, Table I), the total number of sensory axons varies from 366 (cat 162) to 845 (cat 2). The myelinated type of sensory nerve fiber ranges from 252 (cat 162) to 706 (cat 126) and the unmyelinated type of sensory axon varies from 18 (cat 5) to 421 (cat 2). The deafferented petrosal nerve of the dog had

⁴ Foley, J. O., Pepper, H. R., and Kessler, W. H., *J. Comp. Neur.*, 1946, **85**, 141.

⁵ Davenport, H. A., and Barnes, J. F., *Stain Techn.*, 1937, **10**, 139.

960 sensory axons and these included 664 with myelin sheaths and 296 that lacked myelin sheaths.

The greater superficial petrosal nerve of the cat contains from 840 (cat 127) to 2228 (cat 162) motor axons (m, Table I). The myelinated type of motor axon ranges from 445 (cat 127) to 1709 (cat 164). Fewer unmyelinated motor axons, 61 (cat 164) to 719 (cat 161), were found in the greater superficial petrosal nerves of the 9 cats under consideration. The petrosal nerve of the dog had 2527 motor nerve fibers and of these 1635 were myelinated and 892 unmyelinated.

It is apparent from these data and the averages listed in Table I that the motor axons are the predominant functional type of nerve fiber in the greater superficial petrosal nerve of the cat and dog. Although the proportion of total sensory to total motor axons may approach a 1:1 ratio as in cat 127, Table I, where there are 1.05 motor axons for each sensory nerve fiber; yet, on the other hand, there may be as many as 6.08 motor nerve fibers for each sensory axon (cat 162). A similar spread in the ratio between sensory and motor axons occurs when only the myelinated varieties are considered; compare cat 127 with cat 162. There is a greater spread in the ratio of sensory to motor axons when only the unmyelinated variety of axon is considered. There may be a few more sensory unmyelinated nerve fibers (cat 2, 164) or a pronounced excess of unmyelinated motor axons (cat 5). The average numbers of sensory and motor axons of all types (Table I, averages*) show essentially the same proportionate deviation to the motor side. In the single specimen of the dog, the ratio of sensory to motor axons of all varieties approaches a magnitude of 1:3 (Table I, dog 1*).

Discussion. The only comprehensive work on the number of nerve fibers in the normal greater superficial petrosal nerve is that of Van Buskirk.⁶ Van Buskirk listed 252 to 2268 nerve fibers of all types in 13 nerves of the cat, 654 to 3947 axons in 39 counts

of the petrosal nerve of the dog and 862 to 1687 nerve fibers in the greater superficial petrosal nerve of man.

The total number of axons (3487) reported by the author for the normal greater superficial petrosal nerve of the dog (Table I, unoperated side) fits into the range in number of axons which Van Buskirk records for the greater superficial petrosal nerve of the dog.

The enumerations in the intact petrosal nerve of the cat which have been made by the writer (Table I, unoperated side) approximate the maximum number reported by Van Buskirk. In no instance was any count as low as the minimum figure recorded by Van Buskirk. Most of the axon values reported herein for the intact greater superficial petrosal nerve, however, were within 10% of the maximum number given by Van Buskirk for the normal petrosal nerve of the cat. Deviations of this percentile value represent a reasonable check. The explanation for the difference in the minimal and maximal values reported by Van Buskirk and the writer is not apparent; it may be due to normal variation in the nerves of different animals, to the difference in the quality of the silver preparations used by 2 different workers, or it may be a consequence of the different methods used in the 2 investigations for estimating the number of nerve fibers.

There is a poverty of experimentally derived data on the ratio of sensory to motor fibers in the greater superficial petrosal nerve. Of recent interest are the experimental results of Schimert⁷ and Kure and Sano.⁸ Schimert believes that the nerve contains very few if any sensory axons; since he was unable to see any myelinated axons in the greater superficial petrosal nerve of the rat and but few axons in the greater superficial petrosal nerve of the cat after cutting the facial nerve proximally in these animals. Kure and Sano report that the

⁷ Schimert, J., *Z. f. mikr. anat. Forsch.*, 1936, **39**, 35.

⁸ Kure, K., and Sano, T., *Z. f. Zellforsch. u. mikr. Anat.*, 1935, **23**, 495.

⁶ Van Buskirk, C., *J. Comp. Neur.*, 1945, **82**, 393.

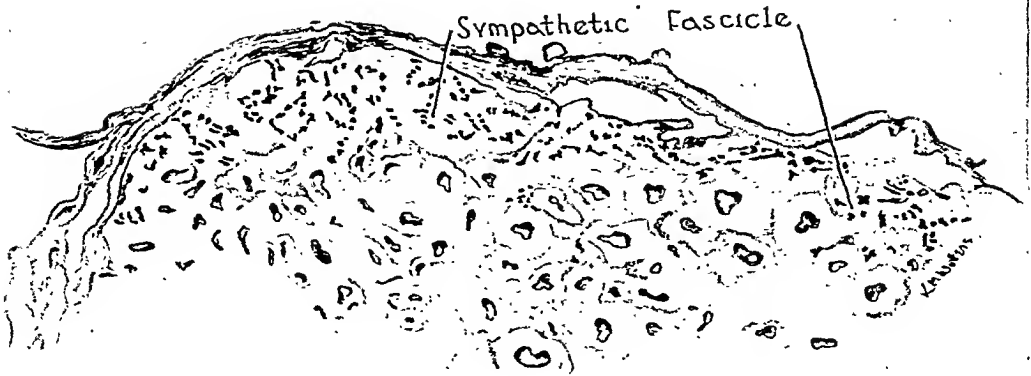


FIG. 1.

From a cross section of the greater superficial petrosal nerve of the cat which has been freed of motor fibers of seventh nerve origin. Attention is directed particularly to the peripherally situated fascicle of sympathetic nerve fibers. Pyridine silver stain.

greater superficial petrosal nerve of the dog contains but few myelinated axons either before (276) or after (145) deletion of its motor nerve fibers by intracranial section of the facial nerve. The observations of these authors are not in accord with the experimental findings of the writer: for, not only do the normal greater superficial petrosal nerves of the cat and dog contain large numbers of myelinated nerve fibers but a sizeable number of myelinated sensory fibers remains in the nerves of both animals (s, Table I, operated side) after the motor fibers have been eliminated.

In our original report³ on the greater superficial petrosal nerve of the cat, it was stated that roughly 62% of the axons of the nerve was motor. Now it is evident that, although the nerves of some cats (cat 126) may contain about this proportion of motor axons, there may be less (cat 127, 51%) or more (cat 162, 85%) motor nerve fibers in the greater superficial petrosal nerve of the cat. The average number of motor nerve fibers in the nerve of the cat (Table I) constitutes about 72% of the total number of nervus intermedius axons in the greater superficial petrosal nerve.

Finally, the proposed ratios (Table I*) between sensory and motor fibers may not

be an exact representation of the ratios of sensory to motor axons which are exclusively of seventh nerve origin. A relatively small number of axons in both the operated and intact nerves may be sympathetic in origin; since, as reported earlier,³ one or more small fascicles of postganglionic sympathetic motor axons may enter the greater superficial petrosal nerve at or just distal to its origin from the geniculate ganglion. These fascicles (Fig. 1) can be readily identified by one familiar with the anatomy of postganglionic bundles and the axons of these aggregations have been omitted in the enumerations which are recorded in Table I. On the other hand, some of the sympathetic nerve fibers may scatter from these bundles⁹ among the intermedius axons of the greater superficial petrosal nerve. Such intermingling would influence the enumerations for total and unmyelinated axons but would not affect the ratios of myelinated sensory and motor nerve fibers since practically all postganglionic sympathetic axons are without demonstrable myelin sheaths.⁹

Summary. The normal greater superficial petrosal nerve of the cat averages 2193 axons and of these, 1625 are myelinated and 568

⁹ Foley, J. O., *J. Comp. Neur.*, 1945, 82, 77.

960 sensory axons and these included 664 with myelin sheaths and 296 that lacked myelin sheaths.

The greater superficial petrosal nerve of the cat contains from 840 (cat 127) to 2228 (cat 162) motor axons (m, Table I). The myelinated type of motor axon ranges from 445 (cat 127) to 1709 (cat 164). Fewer unmyelinated motor axons, 61 (cat 164) to 719 (cat 161), were found in the greater superficial petrosal nerves of the 9 cats under consideration. The petrosal nerve of the dog had 2527 motor nerve fibers and of these 1635 were myelinated and 892 unmyelinated.

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There is a poverty of experimentally derived data on the ratio of sensory to motor fibers in the greater superficial petrosal nerve. Of recent interest are the experimental results of Schimert⁷ and Kure and Sano.⁸ Schimert believes that the nerve contains very few if any sensory axons; since he was unable to see any myelinated axons in the greater superficial petrosal nerve of the rat and but few axons in the greater superficial petrosal nerve of the cat after cutting the facial nerve proximally in these animals. Kure and Sano report that the

⁷ Schimert, J., *Z. f. mikr. anat. Forsch.*, 1936, **39**, 35.

⁸ Kure, K., and Sano, T., *Z. f. Zellforsch. u. mikr. Anat.*, 1935, **23**, 495.

⁶ Van Buskirk, C., *J. Comp. Neur.*, 1945, **82**, 303.

digitoxin, he believed he could detect the presence of as little as 1/100,000 of ouabain and 0.001 mg per cc of digitoxin. He found however, that there was a wide fluctuation in the time of appearance of the arrhythmia at any given concentration of the drug tested.

Although the embryonic chick heart appeared to be suitable for employment in the detection of digitalis, it still was not sufficiently sensitive to give much promise as an aid in the quantitation of the concentration of digitalis in the human body. For this reason, the embryonic duck heart was studied and compared to the chick heart in particular respect to their sensitivity to the digitalis glycoside, Lanatoside C. The results of this study are given in this communication.

Methods. A. The Embryonic Duck Heart. Supposedly fertile duck eggs (White Peking China) were incubated for 88-92 hours at 39°C. They then were transilluminated and the position of the embryo marked on the shell. The shells were then partially removed until the embryo and its vascular sinus were in full view. After the diameter of the sinus had been measured, the total embryo was removed with a sharp scissors and placed in a slide well containing Tyrode's solution. Under a dissecting microscope (12×), the heart was removed from the embryo by means of dissecting, cataract knives. At this stage of development, the heart bulges from the body, just caudal to the head and easily can be identified. It also apparently represents one of the most adhesive, solid tissues of the embryo for it may be separated in its entirety by simple teasing of embryonic tissue lying adjacent to it. After separation, the heart is floated gently upon a small spatula and transferred to a second slide well containing 0.1 cc of the solution to be tested. This second slide rests on the stage of a compound microscope which is in a box automatically maintained at 33°C. by means of an electric lamp of 50 watts in circuit with a mercury thermostat and vacuum tube relay switch. After this second transfer, the heart is observed through the microscope (30×) and (1) strength and rate of contraction, (2) occurrence of an arrhythmia

and (3) duration of beating are noted.

It was found after preliminary studies that those hearts of embryos contained in vascular sinuses measuring from 20-30 mm in diameter were most suitable for the detection of the digitalis glycoside employed.

Lanatoside C, the glycoside of digitalis lanata, was used in all experiments. An ampule containing 0.2 mg per cc was opened for each day's experiments. Dilutions of this drug varying from 0.001 to 0.00001 mg per cc were studied, after a control series of embryonic hearts were immersed in simple Tyrode's solution and studied.

B. The Embryonic Chick Heart. Embryonic chick hearts were obtained and studied in the same manner except that (1) they were incubated only 66 hours at 38.5°C and (2) no embryo having a sinus diameter greater than 30 mm was used.

Results. A. The Embryonic Duck Heart. The embryonic duck heart was found to be extraordinarily sensitive to the digitalis glycoside employed. The first indication of this sensitivity was an acceleration and an intensification in the vigor of cardiac contractions. Then, just prior to the occurrence of auriculoventricular block (which was used as the actual indicator for the presence of digitalis) the heart was observed to contract irregularly, very rapidly, jerkily and weakly. The auriculoventricular block always began as partial A-V block and progressed to complete block. Concomitant with the progression of the block, the irregularity of the rate of beating became more manifest. Finally, if sufficient concentrations of digitalis were used, the heart stopped beating in systole.

Thus it was found (Table I A) that when 23 embryonic duck hearts were placed in 0.1 cc of a solution containing 0.001 mg of the glycoside per cc, each began to accelerate and all began to demonstrate auriculoventricular block after an average period of 12 minutes (range: 6 to 28 minutes). They ceased to beat after an average period of 37 minutes (range: 29 to 62 minutes). Approximately the same phenomena were observed when 15 hearts were placed in 0.1 cc of a

are without myelin sheaths. A single intact nerve of the dog held 3487 nerve fibers, which were divided into 2299 myelinated and 1188 unmyelinated axons.

An average of 597 sensory nerve fibers remains in the greater superficial petrosal nerve of the cat after its motor axons have been eliminated. Of this average number of sensory nerve fibers, 461 are myelinated and 136 are unmyelinated. The deafferented petrosal nerve of one dog contained 960 sensory axons and 664 of these were myelinated while 296 were without myelin sheaths.

It is estimated that the greater superficial petrosal nerve of the cat has an average of approximately 1596 motor nerve fibers and these are divided into 1164 myelinated and 432 unmyelinated nerve fibers. The greater superficial petrosal nerve of the dog contained 2527 motor axons and 1635 of these were myelinated and 892 were unmyelinated.

It is evident, therefore, that the majority of the axons of the greater superficial petrosal nerve are motor nerve fibers, there being an average of 2 plus motor fibers for each sensory axon.

15732

Employment of the Embryonic Duck Heart for the Detection of Minute Amounts of a Digitalis Glycoside* (Lanatoside C).

MEYER FRIEDMAN AND RENE BINE, JR.

From the Harold Brunn Institute for Cardiovascular Research, Mount Zion Hospital, San Francisco, Calif.

Exact quantitative information concerning the absorption and excretion (or destruction) of digitalis in the human body is still to be obtained. Such knowledge has not been gained primarily because of the absence of any test sufficiently sensitive to detect the relatively minute amounts of digitalis which must be present in the tissues of patients receiving digitalis. The use of the frog, cat and pigeon for the assay of digitalis has been limited to the quantitation of relatively large quantities of the drug and cannot be utilized for the demonstration of digitalis if the latter is present in but fractions of a microgram.

Pickering¹ was the first to employ the embryonic chick heart for the detection of digitalis. Using embryos between 60-75 hours of age, he demonstrated the striking similarity of such hearts to that of the adult

mammal, especially in regard to their response to digitalin and strophanthin. He found that the embryonic chick heart would cease beating after the injection of 0.012 mg of digitalis *in situ*. Lagen and Sampson² applied digifoline directly upon the embryonic heart of the chick and were able to demonstrate an electrocardiographic effect after such a procedure. Hall³ also employed the embryonic chick heart for the assay of crude digitalis. Paff⁴⁻⁶ dissected the heart from the chick embryo and immersed it in the solution containing the drug to be tested. Using the occurrence of an arrhythmia as a test for the presence of both ouabain and

² Lagen, J. B., and Sampson, J. J., *Proc. Soc. Exp. Biol. and Med.*, 1932, **29**, 735.

³ Hall, E. M., *Am. J. Pharm.*, 1932, **104**, 310.

⁴ Paff, G. H., *J. Pharm. and Exp. Therap.*, 1932, **69**, 311.

⁵ Paff, G. H., and Johnson, J. R., *Am. J. Physiol.*, 1938, **126**, 753.

⁶ Paff, G. H., *J. Pharm. and Exp. Therap.*, 1940, **70**, 235.

* Aided by a grant from the Dazian Foundation for Medical Research and by a grant from the Sandoz Chemical Works, Inc.

¹ Pickering, J. W., *J. Physiol.*, 1893, **14**, 383.

Conclusion. The embryonic duck heart, similar to the embryonic chick heart was found to exhibit sensitivity to the action of a digitalis glycoside as characterized by alteration in rate, rhythm and force of contraction. Moreover the embryonic duck

heart was able to be used to detect as little as one two-hundredth of a microgram of the digitalis glycoside (Lanatoside C.) This is thought to represent the most sensitive indicator now available for the presence of a digitalis glycoside.

15733 P

Isolation of Pleuropneumonia-like Organisms from Pathological Specimens with the Aid of Penicillin.*

L. DIENES.

From the Department of Pathology and Bacteriology, the Massachusetts General Hospital, and the Robert W. Lovett Memorial Foundation, Harvard Medical School, Boston, Mass.

Pleuropneumonia-like organisms (P.P.L.O.) were isolated from the genito-urinary tract of human patients and in rare cases from other locations.¹⁻⁵ Such organisms are often present in the vagina or cervix without apparent connection with any disease. In males they occur only in pathological conditions, and, according to observations collected in the last few years, they play a noticeable role as causative agents in urethritis, prostatitis and cystitis. They have been isolated from cases of severe cystitis which were repeatedly negative for the usual bacteria. Infection of the genito-urinary tract was complicated in about 30% of the cases with acute polyarthritis. It is essential in studying the role of these organisms in human disease that the methods used for their culture and identification be free from error.

The presence of P.P.L.O. can be proven only by cultivation. They grow on media enriched with human or animal serum or with ascitic fluid. Large colonies (0.1-0.3 mm) can be identified with the low power of the microscope. If the colonies remain small (.01-0.1 mm) as often happens in cultures from pathological specimens, they cannot be identified by this method. The agar fixation method which Klieneberger⁴ and Salaman⁵ used utilizes the impression left by the culture on a coverslip. Small colonies do not adhere to the glass and can not be recognized. This method is subject to error even in the case of large colonies. They are identified by the round bodies which develop on the surface layer and it is not unusual for bacteria to produce similar large bodies. The most reliable method for the identification of the P.P.L.O. is the use of stained agar preparations.⁶ A square of the agar culture is cut out and is then covered with a coverslip carrying the stain. The colonies are present in their entirety in the preparations and can be identified with certainty.

Demonstration of P.P.L.O. is usually not possible in the presence of abundant bacterial growth. The P.P.L.O. are resistant to sulfonamides and to penicillin both of which were recommended to suppress bacterial growth. Salaman used penicillin cups for this purpose.⁵ The author can confirm the

* The expenses of this investigation have been defrayed in part by a grant from the Commonwealth Fund.

This is publication No. 91 of the Robert W. Lovett Memorial Foundation for the study of crippling disease.

¹ Dienes, L., *Proc. Soc. Exp. Biol. and Med.*, 1940, **44**, 470.

² Dienes, L., and Smith, W. E., *Proc. Soc. Exp. Biol. and Med.*, 1942, **50**, 99.

³ Beveridge, W. J. B., *Med. J. Australia*, 1943, **2**, 479.

⁴ Klieneberger-Nobel, E., *The Lancet*, 1945, **2**, 46.

⁵ Salaman, M. H., and collaborators, *J. Path. Bact.*, 1946, **58**, 31.

⁶ Dienes, L., *J. Inf. Diseases*, 1939, **65**, 24.

TABLE I.
Effect of Digitalis on the Embryonic Duck and Chick Heart.

Digitalis (mg/cc)	Avg sinus diameter (mm)	Hearts (No.)	A-V block (No.)	Onset- A-V block (min.)	Duration of beating (min.)	Avg rate	Type of Contractions
A. Embryonic Duck Heart.							
0 (control)	30	15	0	—	>60	59	Moderate
.001	31	23	23	12	37	85	Vigorous
.0005	33	15	15	15	42	82	"
.0001	29	15	15	35	54	76	"
.00005	32	18	14	39	>60	73	"
.00001	28	16	0	—	>60	61	Moderate
B. Embryonic Chick Heart.							
0 (control)	27	10	0	—	>60	76	"
.001	21	25	21	15	41	96	Vigorous
.0005	23	14	10	18	>60	96	"
.0001	17	10	0	—	>60	82	Moderate

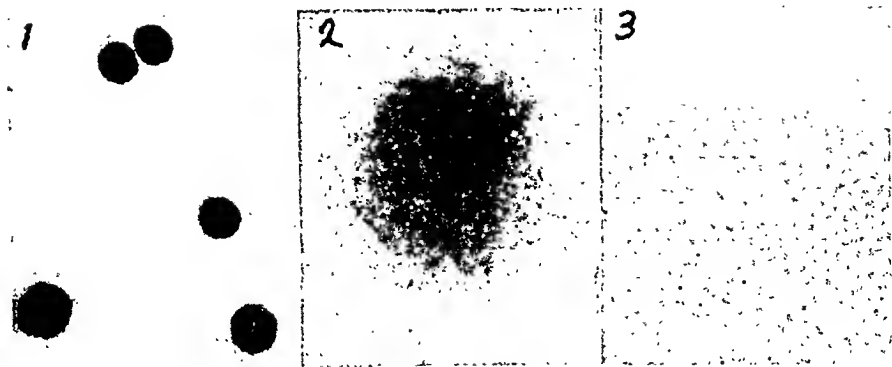
solution containing 0.0005 mg of glycoside per cc (Table I A) except that onset of auriculoventricular block occurred somewhat later (average: 15 minutes) and the hearts continued to beat somewhat longer (average: 42 minutes). When 15 hearts were placed in a solution containing 0.0001 mg of glycoside per cc, auriculoventricular block occurred in all of them (Table I A) but the average time of onset was 35 minutes. These latter hearts also continued to contract for an average period of 54 minutes (range: 43 to 71 minutes). Auriculoventricular block occurred in 14 of 18 hearts (78%) placed in a solution containing 0.00005 mg of glycoside per cc after an average period of 39 minutes (range: 24 to 68 minutes). The longevity of these hearts was not affected however by the concentration of glycoside employed. No auriculoventricular block occurred in any of 16 embryonic hearts immersed in a solution containing 0.00001 mg of glycoside per cc (Table I A).

B. The Embryonic Chick Heart. As can be seen by inspection of Table I B, the action of the digitalis glycoside on the embryonic chick heart was similar to its effect upon the duck heart except that the former type of heart was much less sensitive to the drug. Thus (Table I B) whereas a concentration of as little as 0.00005 mg of glycoside per cc could be detected by the use of the duck heart preparation, only 0.0005 mg per cc could be detected by the chick

heart. Furthermore, even as much of the digitalis glycoside as 0.001 mg per cc could not be invariably detected by using the chick heart method.

Discussion. The above results indicated that the embryonic duck heart was far more sensitive to the digitalis glycoside employed than the embryonic chick heart. By the former's employment, 0.1 cc of a one to 20 millionth dilution of Lanatoside C. (0.000005 mg) could be detected. It is believed that this represents the most sensitive indicator for the presence of a digitalis glycoside yet described.

The embryonic heart of the duck also has certain advantages for its use in the study of cardiac physiology. Pickering¹ earlier stressed the similarity of the chick heart to the mammalian heart and it is most likely that the duck heart also has the same similarities. There are no nervous elements in the embryonic heart at this stage,¹ a state of affairs which allows the study of cardiac musculature alone. Furthermore, the ability of this type of heart to contract regularly over a period of at least 60 minutes in very small quantities of fluid, allows the possibility of testing samples, meager in amount. Finally, because of the relative paucity of tissue composing the heart, more opportunity is allowed for changing the ionic milieu of the cardiac cells (when desired) than might be possible were an adult mammalian heart the subject of experimentation.



Photograph 1 shows well-developed colonies of the P.P.L.O. isolated from *H. influenzae* ($\times 90$).

Photograph 2 shows a medium-sized colony with high magnification ($\times 3000$). The colony is embedded in the agar and the shape of the individual organisms is not clearly defined.

Photograph 3 shows the edge of the colony of the parent *H. influenzae* strain ($\times 3000$). The organisms are larger than in Photograph 2 and have a sharp contour.

Photographs 1 and 2 were made from stained agar preparations; No. 3 was made from an impression preparation after agar fixation.

ance or develop into tiny colonies resembling the organisms of bovine pleuropneumonia. The pleuropneumonia-like (P.P.L.) growth of *H. influenzae* remained very small, and it could not be determined whether or not they were able to grow indefinitely since they were always overgrown by regular bacterial forms.

Pierce³ has observed that penicillin facilitates the isolation of L_1 from *Streptobacillus m.* by the elimination of the regular bacterial forms. After some experimentation with penicillin a procedure was found by which pleuropneumonia-like organisms (P.P.L.O.) can be isolated from cultures of *H. influenzae* and grown in pure culture.

H. influenzae is planted heavily on blood agar plates. One or several small troughs are made on the agar without cutting through it, and in each is placed a drop of solution containing 2000 units of penicillin per ml. After absorption of the penicillin solution the plates are made anaerobic by growing *B. prodigiosus* together with the culture and sealing the petri dish with paraffin. The plates are incubated at 30°C and 36°C and are opened after 1, 2, 3, and 4 days. Bacterial growth is completely inhibited in an

area 2 to 3 cm in diameter around the trough. It is apparent in stained agar preparations that in the area of inhibition the originally deposited bacteria swell up into large round bodies sometimes as large as 15 to 20 micra without any indication of multiplication. In the border area where growth becomes apparent the organisms are swollen in a similar way. Further out they are replaced first by filaments and then by bacilli of the usual appearance. The swollen organisms do not show any sign of development if they are transplanted. However P.P.L. growth begins to develop from a few large bodies in the area in which bacterial growth is inhibited. Often after 48 hours many P.P.L. colonies are visible in stained agar preparations, and after 3-4 days a crop of tiny colonies, visible with the hand lens, grows in the zone of inhibition. These colonies are embedded in the agar and correspond in appearance and morphology to the organisms of the pleuropneumonia group.

The tiny colonies can be transplanted by cutting out a piece of agar containing them and smearing it on blood agar and boiled blood ascitic agar plates. The plates are treated with penicillin as were the original ones and are incubated anaerobically with *B. prodigiosus*. The cultures later can be grown

³ Pierce, C. H., personal communications.

⁴ Dienes, L., Proc. Soc. Exp. Biol. and Med., 1940, 44, 470.

effectiveness of penicillin, but without necessary precautions its use leads to error and will confuse the study of P.P.L.O.

One source of error is that in the area of inhibition, the colonies may remain very small, and the organisms may swell up into large round bodies. They can not be distinguished in an impression preparation from colonies of P.P.L.O. It is probable that the colonies which Salaman identified as mixed colonies of Gonococci and P.P.L.O. were such altered Gonococcus colonies. The author has never seen P.P.L.O. and Gonococcus together in cultures from male patients.

This source of error can be eliminated by using stained agar preparations in which penicillin does not interfere with the identification of P.P.L.O.

The second source of error is the fact that P.P.L.O. can develop from bacteria under the influence of penicillin. It has been shown in another paper that this occurs in pure culture of *H. influenzae*.⁷ From a series of 14 throat cultures which were recently studied, P.P.L.O. developed in 10 in the vicinity of the penicillin cups. The distribution of the colonies indicated that they were produced by the penicillin. They were situ-

ated in the area of inhibition and were absent in the area not exposed to penicillin. Colonies of P.P.L.O. never develop in throat cultures without the use of penicillin, while they grow very well together with bacteria in cultures from the genito-urinary tract.

According to these observations, penicillin can be used only to screen the specimens for the presence of P.P.L.O. In order to be sure that they are present in the specimens and are not produced from bacteria, their colonies must be seen also in cultures not treated with penicillin. The P.P.L. strains isolated from human patients form a heterologous group, and it is important to distinguish those which are connected with bacteria from those which, like the animal pathogens, do not show such connection.

Summary. Penicillin is of considerable help in isolating pleuropneumonia-like organisms from specimens contaminated with bacteria. Penicillin alters the colonies of certain bacteria in such a manner that in impression preparations they become indistinguishable from pleuropneumonia-like organisms. Furthermore penicillin may induce the growth of pleuropneumonia-like organisms from bacteria. To prove that these organisms are present in the specimens, the characteristic colonies must be apparent in the cultures without the use of penicillin.

⁷ Dienes, L., *Proc. Soc. Exp. Biol. and Med.*, in press.

15734 P

Isolation of Pleuropneumonia-like Organisms from *H. influenzae* with the Aid of Penicillin.*

L. DIENES.

From the Department of Pathology and Bacteriology, the Massachusetts General Hospital, and the Robert W. Lovett Memorial Foundation, Harvard Medical School, Boston, Mass.

It has been previously noted¹ that certain

* The expenses of this investigation have been defrayed in part by a grant from the Commonwealth Fund.

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¹ Dienes, L., *J. Bacteriology*, 1942, **44**, 37.

strains of *H. influenzae* show a pleomorphism similar to that observed in *Streptobacillus moniliformis*¹ and bacteroides.² The bacilli swell up into large round bodies which either disintegrate into bacteria of usual appear-

² Dienes, L., and Smith, W. E., *J. Bacteriology*, 1944, **48**, 125.

Infectivity of *Trypanosoma cruzi* after Cultivation for Thirteen Years in vitro Without Animal Passage.

A. PACKCHANIAN AND H. H. SWEETS, JR.

From the School of Medicine, University of Texas, Galveston, Texas.

It is an established fact that when *Trypanosoma lewisi* is cultured on N.N. (Novy and MacNeal) medium¹ and subcultured at 3- or 4-week intervals the strain remains infective to rats during the first year of cultivation. However, at the end of 2 years' *in vitro* cultivation the strain loses its ability to infect rats, even if a massive inoculum prepared from several cultures is injected into young or splenectomized rats.²⁻⁴

The question whether or not *Trypanosoma cruzi* also becomes attenuated after a similar period of *in vitro* cultivation had not been investigated previous to this study. Since we have in our possession a strain of *Tr. cruzi* which has been kept *in vitro* for nearly 13 years without animal passage, this problem was studied.

Methods and Materials. A naturally infected *Triatoma geniculatus* received from Panama was crushed and the intestinal contents of the insect were inoculated into a guinea pig. On June 20, 1932, a strain of *Tr. cruzi* was cultured from the guinea pig's blood on N.N. medium.⁴ This culture has been maintained on N.N. medium at room temperature or at 25°C. Subcultures were made usually every 4 to 8 weeks; occasionally a longer interval was allowed to elapse before transfers were made.⁵ During this 13-year period, the strain was subcultured 81 times without any animal passage.

The 81st subculture of this strain of *Tr. cruzi* was inoculated to 8 mice (*Mus musculus*). The blood of these animals was examined microscopically at various intervals following inoculation, devoting about 5 minutes to each preparation. Xenodiagnosis (which consists of feeding normal *Triatoma* on the animals and subsequently examining the insect for infection with flagellates), was used on 2 mice. At varying times after inoculation, the mice were sacrificed and the blood was inoculated to N.N. medium. The tubes were covered with rubber caps, incubated at 25°C and examined after about 3 weeks for motile flagellates.

Results. The trypanosomes were demonstrated by direct microscopic examination in 4 out of 8 mice from 27 to 48 days after inoculation.

One of the xenodiagnostic tests was positive 50 days after injection, while the other was found negative 46 days following inoculation.

The mice were killed from 34 to 53 days after inoculation, and the blood of all 8 animals gave positive cultures for flagellates.

Summary. A strain of *Trypanosoma cruzi* cultured from experimentally-infected guinea pig on N.N. medium was subcultured at various intervals on N.N. medium for 13 years without animal passage. At the end of this period the strain was found to be still capable of producing infection in experimental animals.

The presence of *Tr. cruzi* was microscopically demonstrated in the circulating blood of 50% of the inoculated animals. However, cultures made from the heart blood of all the animals on N.N. medium were positive for *Tr. cruzi*.

¹ Novy, F. G., and MacNeal, W. J., *J. Infect. Dis.*, 1904, 1, 1.

² Novy, F. G., Perkins, W. A., and Chambers, R., *J. Infect. Dis.*, 1912, 11, 411.

³ Behrens, C. A., *J. Infect. Dis.*, 1914, 15, 24.

⁴ Packebanian, A., *Science*, 1934, 80, 407.

⁵ Packebanian, A., *J. Parasitol.*, 1943, 29, 275.

without penicillin.

The culture so obtained differs from the parent organism in the following characteristics: (1) The organisms in young cultures are much smaller. They do not have a sharp contour like usual bacilli and they are very soft. The slightest injury deforms or destroys them. (2) The colonies do not grow on the surface of the agar but extend below the surface. The well-developed colonies as visible in Fig. 1 have the appearance typical of the pleuropneumonia group. (3) In older colonies the organisms swell into round bodies. (4) The organisms do not show satellite growth and are very resistant to penicillin. In appearance, staining and physical properties these organisms are very similar to the L_1 isolated from *Streptobacillus moniliformis*, to the corresponding organism isolated from bacteroides and to the whole pleuropneumonia group.

Five strains of *H. influenzae* were studied by the technic described. The strain from which the P.P.L.O. were most easily isolated was cultured from the sputum of a case of bronchiectasis treated with penicillin aerosol. The second strain, an *H. influenzae* Type B, from which large colonies of P.P.L.O. developed but which could not be kept in continuous cultivation was isolated from a blood stream infection. Immediately after isolation this culture was pleomorphic and produced tiny P.P.L. colonies. This property had been lost by the time experiments with penicillin were started. The other strains were not pleomorphic. Two, isolated from blood and from the conjunctiva respectively, produced a few fairly large P.P.L. colonies

in the area of inhibition. The third strain, isolated from a sputum did not produce P.P.L. colonies. The strains with the exception of the second were studied immediately after isolation. Most freshly isolated strains of *H. influenzae* apparently can be induced to produce P.P.L.O. by exposure to penicillin. There is considerable variability among the strains concerning the ease with which P.P.L.O. are produced and with which they can be kept in cultivation. A similar variability has been observed in the viability of P.P.L.O. isolated from different bacteroides strains.² Use of the same technic has thus far not been effective in isolating P.P.L.O. from gonococci, *E. coli* and several strains of streptococci.

The role of penicillin in the growth of P.P.L.O. is unknown. It is possible that it consists mainly in the elimination of regular bacterial forms. The fact that swelling of bacteria induced by penicillin is very similar to the swelling occurring in naturally pleomorphic strains, may indicate that the effect of penicillin is more complex. The growth of P.P.L.O. does not seem to be a degenerative process because a viable organism, closely similar to an important group of pathogens, is produced.

Summary. With the aid of penicillin a strain of pleuropneumonia-like organism has been isolated from a culture of *H. influenzae*. This strain could be kept in continuous cultivation. Colonies of similar organisms were seen in 3 other cultures of *H. influenzae*, but their propagation in pure culture was not successful.

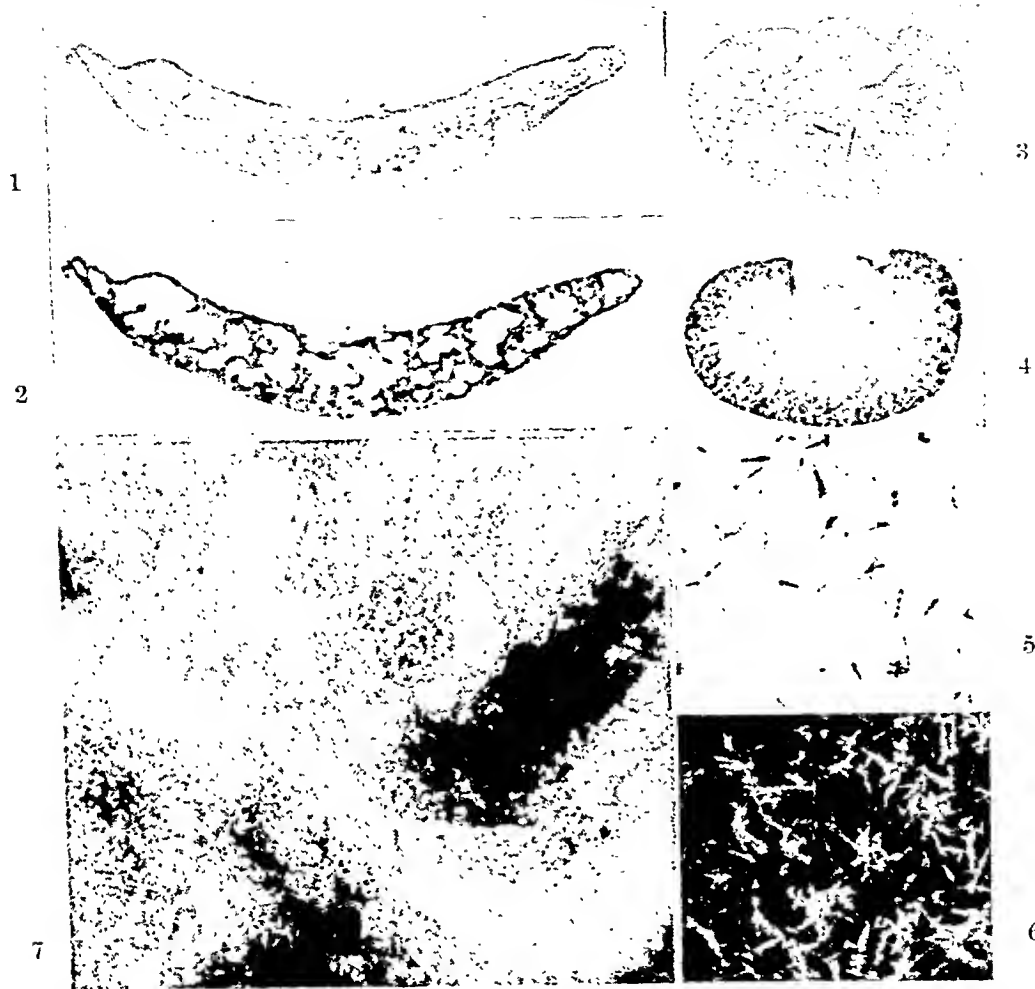


PLATE I.

1. Photomicrograph of mouse spleen containing polonium, hematoxylin, and eosin. $\times 5$.
2. Temporary contact autoradiograph prepared from (1). $\times 5$.
3. Photomicrograph of mouse kidney containing polonium.
4. Temporary contact autoradiograph prepared from (3).
- 5 and 6. Dark field views of alpha particle tracks from an area of low and medium polonium content (salivary gland). $\times 400$.
7. Photomicrograph of permanent tissue-photographic emulsion preparation of mouse kidney containing polonium showing renal cortex with extremely dense areas of alpha particle tracks beneath distal convoluted tubules. $\times 500$.

justment knob of the microscope, one can trace each alpha particle track from its end to its beginning and thus to the precise point in the tissue from which it originated. It is thus possible to determine whether the parent atom was located in cytoplasm or nucleus of a given cell. The cellular distribution of radioelements which decay solely with emission of beta or gamma radiations probably cannot be studied by such methods.

Macro images for low power photomicrography are best prepared by one of two techniques in which the tissue is not mounted on the emulsion permanently. The tissue ribbon may be mounted on a glass slide, deparaffinized in xylene, coated with celloidin, and placed in contact with the photographic emulsion. At the end of the exposure the glass slide and the photographic plate are separated, the plate being developed and the

Microscopic Historadiographic Technic for Locating and Quantitating Radioactive Elements in Tissues.

K. M. ENDICOTT AND HERMAN YAGODA. (Introduced by F. S. Daft.)

From the Pathology Laboratory and the Industrial Hygiene Research Laboratory, National Institute of Health, Bethesda, Md.

Accurate quantitative microscopic studies of tissue and cellular distribution of radioactive elements which decay with emission of alpha particles may be made quite easily by the technics to be described. The essential points in the technic are (1) the use of fine-grained silver-bromide emulsions with selective response to alpha radiation,^{1,2} (2) the mounting of tissue sections permanently on the emulsion at the beginning of the exposure, (3) examination of such preparations at 400 diameters in dark-field illumination for determination of number of alpha particle tracks per unit area of emulsion, and (4) examination of such preparations at 1000-2000 diameters for determination of the precise cellular location of the radioelement by tracing straight tracks through the emulsion to their points of origin in the tissue.

The following technics have been evolved in the study of radium F (polonium) which decays with emission of alpha particles and very low intensity gamma rays. The polonium is administered in physiological saline buffered with NaHCO_3 . Tissues are fixed in buffered neutral 4% aqueous HCHO for 48 hours. Bones are decalcified in 5% aqueous HCOOH . Tissue blocks are dehydrated in acetone, cleared in gasoline, embedded in paraffin in a vacuum oven and sectioned at 5 μ . The ribbons are floated off cold water onto the photographic emulsion (Eastman alpha particle plate No. 329,489). All manipulations of the undeveloped photographic plates are carried out in a room illuminated by the appropriate safelight. The preparation is dried quickly in a current of air and is placed in a light-tight container for the remainder of the exposure period. At the end of the exposure period the paraffin is removed by placing the plate in xylene

for 5 minutes and the plate is dried in a current of air. The plate is developed in D-19, hardened in SB-4, and fixed in F-5 (Eastman Kodak formulae) at room temperature after which it is washed in filtered tap water for 2 hours. The plate may be stained immediately or dried and stored for staining later. The plate is stained in freshly prepared Weigert's acid-iron hematoxylin for 2 minutes, washed in tap water 5 minutes, dehydrated in 3 changes of acetone, cleared in 1:1 acetone-xylene and in 3 changes of xylene. A drop of xylene-clarite is placed on the tissue and the preparation is covered with a glass coverslip.

Crude quantitation of the relative distribution of polonium in tissues may be obtained by microdensitometer analysis of the developed emulsion. A much more precise local analysis is obtained by counting the number of particle tracks per unit area of emulsion. The amount of polonium present is directly proportional to the number of tracks when the experimental conditions are constant. Determination of relative distribution is thus reduced to comparison of number of tracks. The actual content of polonium per unit of tissue can be calculated from decay constant, length of exposure, spatial relationship of emulsion and tissue, volume of tissue, etc., but this calculation is not ordinarily necessary.

In addition to the quantitative aspects, there is another feature of great interest to the biologist. This is at present limited to the alpha ray emitting elements. In preparations in which the tissue is mounted permanently on the emulsion at the beginning of the exposure, one can locate the point of origin of the alpha particle with great precision. This depends upon the extremely shallow depth of focus at high magnifications (500-2000 \times). By turning the fine ad-

¹ Yagoda, H., *Am. Mineralogist*, 1946, **31**, 87.

² Yagoda, H., *Am. Mineralogist*, 1946, **31**, 462.

15737 P

Infection of Mice with Tubercle Bacilli Grown in Tween-Albumin Liquid Medium.

CYNTHIA PIERCE, RENE J. DUBOS, AND GARDNER MIDDLEBROOK.

From the Laboratories of the Rockefeller Institute for Medical Research, New York City.

Tubercle bacilli, growing diffusely in liquid media containing a water dispersible ester of oleic acid (Tween 80), retain unaltered many of their morphological and biological properties.^{1,2} The present paper describes the virulence of these cultures for mice of different genetic backgrounds inoculated under various experimental conditions. The cultures used in the infection tests to be reported were grown for 7-10 days in a medium containing 0.05% Tween 80 and 0.2% bovine albumin (serum Fraction V).² Macroscopically, these cultures appear homogeneous, but in reality consist of microscopical clumps. Their density corresponds to approximately 0.20 mg/cc in terms of dry weight of bacilli.

Mice, 3 to 6 weeks old, of the Rockefeller Institute strain inoculated intravenously with 0.01 cc of whole culture begin to lose weight during the second week after infection and die in 3 to 4 weeks with a disease primarily pulmonary. The pulmonary lesions consist of discrete and confluent nodules varying in size, pearly gray in color and firm in consistency. Stained microscopic sections show innumerable tubercle bacilli in these areas. Heart muscle is often infiltrated with very many small lesions. When the inoculum is reduced 10-fold to 0.001 cc, only an occasional animal dies after the 4th week of infection, although all animals sacrificed at this time show pulmonary lesions.

Much larger amounts of culture (0.5-1.0 cc) are required to produce death within 3 to 4 weeks when the infective dose is introduced by the intraperitoneal route. This minimal lethal dose can be reduced apprecia-

bly by adding fresh egg yolk to the bacterial suspension.

Fresh egg yolk is diluted with an equal part of 0.85% saline. One volume of culture is emulsified with one volume of the diluted egg yolk suspension immediately prior to infection. The data presented in Table I illustrate the enhancement of infection by the addition of fresh egg yolk to the culture.

The infection tests just described were carried out with the classical H37Rv culture obtained through the courtesy of Mr. William Steenken of the Trudeau laboratory. Similar results have been obtained with other human and bovine cultures of tubercle bacilli recently isolated from pathological materials. On the other hand, saprophytic acid-fast bacilli and variants derived from pathogenic strains but known to be devoid of pathogenicity for guinea pigs and chick embryos, fail to establish a progressive disease in mice even when injected in large amounts. Table II illustrates the results of inoculating mice (Rockefeller Institute strain) by the intravenous route with 2 different variants of the H37 culture of human tubercle bacilli, H37 (virulent) and H37Ra (avirulent). Even with the addition of egg yolk, 0.2 cc of H37Ra culture fails to establish a progressive infection or to produce grossly visible lesions.

Fifteen different strains of mice have been compared for susceptibility to tuberculous infection by the intravenous and intraperitoneal routes. The progress of the disease was determined by weight changes of the animals at weekly intervals, length of survival, number and extent of macroscopic pulmonary lesions, and enlargement of the spleen. Whatever the mode of infection, and whatever the criteria used for evaluating the severity of the disease, the differences observed are consistent and permit the recognition of marked

¹ Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, 83, 409.

² Dubos, R. J., Davis, B. D., Middlebrook, G., and Pierce, C., *Am. Rev. Tuberc.*, 1946, 54, 204.

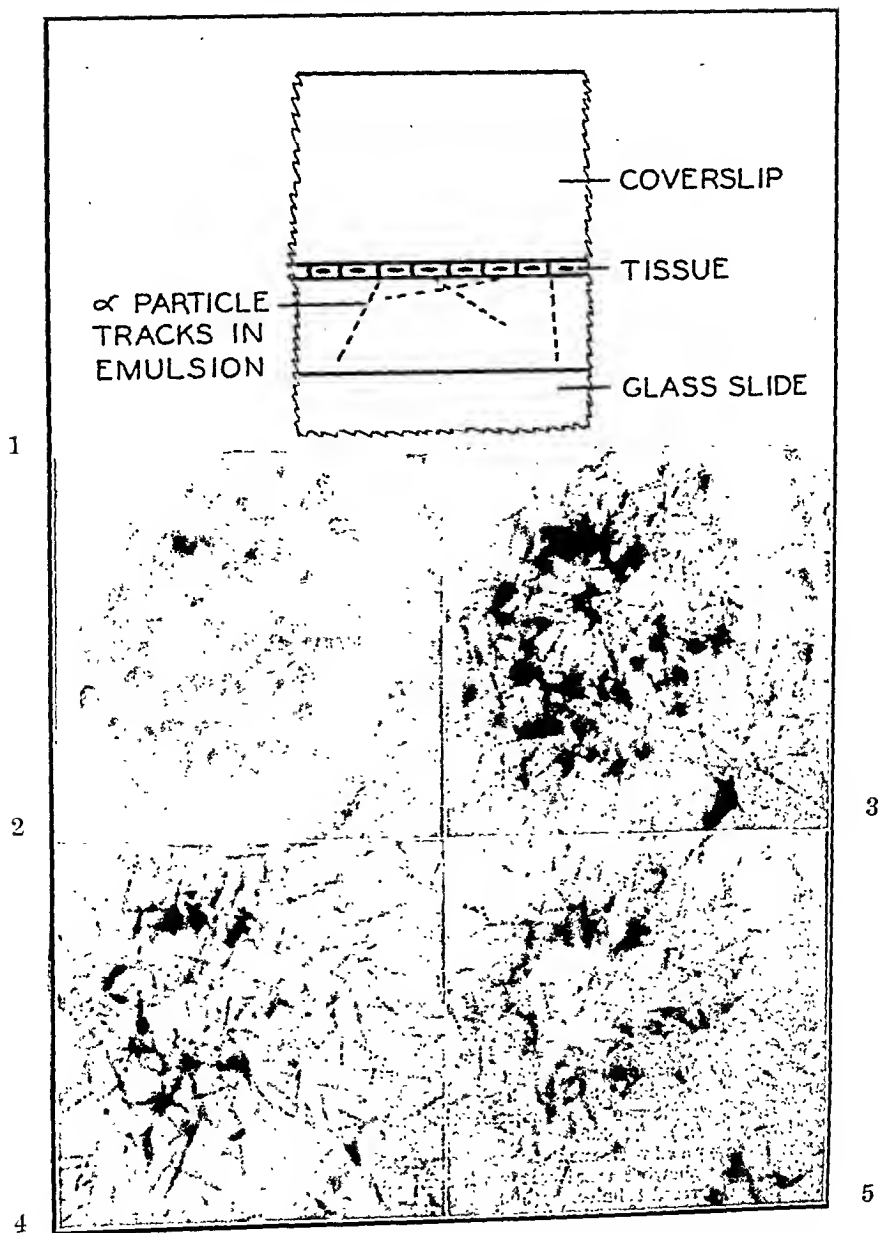


PLATE II.

1. Schematic drawing of permanent tissue-photographic emulsion preparation.
 2, 3, 4, 5. Photomicrographs of renal glomerular tuft and underlying photographic emulsion at successively deeper focus. Permanent tissue-photographic emulsion preparation. Hematoxylin and eosin stain. $\times 1000$.

tissue slide being stained by any one of the many ordinary methods after removing the celloidin with acetone. Equally satisfactory and in many cases superior historadiographs are obtained if the plane-surfaced paraffin

block, which remains after the sections are cut, is placed in contact with the photographic plate for preparation of the historadiograph. The image is compared with the stained section last cut from the block.

TABLE I.
Analyses of the Electrophoretic Patterns of Serum and Pleural Effusion After Lethal Doses of ANTU.

Hr after ingestion	% area				Mg N %			
	Albumin + α_1	$\alpha_2 + \alpha_3$	$\beta_1 + \beta_2$	γ	Albumin + α_1	$\alpha_2 + \alpha_3$	$\beta_1 + \beta_2$	γ
75 mg/kg ANTU in gelatin capsule by mouth.								
Dog 1: Wt—27.3 kg.								
	Serum.							
Control	51	14	35		492	135	338	
4	49	17	34		480	166	333	
6	48	16	36		446	149	335	
10	48	21	32		385	168	257	
12	45	24	32		373	199	265	
13½	47	18	35		307	118	229	
15	43	22	34		279	143	221	
	Pleural Effusion.							
15	54	12	34		388	86	244	
Dog 2: Wt—20.6 kg.								
	Serum.							
Control	47	16	30	5	540	184	345	58
7	46	12	34	6	520	136	384	68
14	45	13	35	6	476	138	371	64
15	46	15	33	6	527	172	378	69
16	47	13	34	7	446	124	323	67
17	52	10	31	7	505	97	300	68
	Pleural Effusion.							
15	46	12	36	5	324	85	254	35
16	47	15	38		329	105	266	
17	53	10	37		366	69	255	
Dog 3: Wt—20 kg.								
700 mg ANTU intravenously injected in a 6% urocia-saline mixture.								
	Serum.							
0	40	19	33	8	440	209	363	88
6	40	18	33	9	388	175	320	87
7	41	18	35	6	400	176	341	58
7½	37	23	31	9	396	246	332	96
	Pleural Effusion.							
6	49	13	38		318	85	247	
7½	49	10	34	7	360	74	250	52
Dog 4: Wt—15.9 kg.								
560 mg ANTU injected intravenously.								
	Serum.							
0	47	14	32	7	545	162	371	81
7	39	19	33	9	392	177	307	84
	Pleural Effusion.							
7	53	12	28	7	379	86	200	50

Richter also observed that if a rat ingests a sublethal dose of ANTU, its tolerance for larger amounts develops rapidly and persists for a comparatively long period. The thiourea portion of the ANTU molecule probably affects the thyroid metabolism of these animals. A number of investigators have found that thiourea and its derivatives depress the functional activity of the thyroid gland by interfering with the synthesis of thyroxine and diiodotyrosine.^{2,3} In man,

thiourea administration causes a rise of blood cholesterol⁴ which may be expected in hypothyroidism. Moore, Levin, and Smelser⁵ showed that an α -globulin component of rat sera increases after thyroidectomy or after thiouracil feeding.

This report deals with (a) the protein and cholesterol concentrations of the serum and pleural effusion in dogs dying after oral or intravenous administration of ANTU; and

⁴ Jennings, L. M., Mawson, C. A., and Tindall, W. J., *Lancet*, 1944, 2, 91.

⁵ Moore, D. H., Levin, L., and Smelser, G. K., *J. Biol. Chem.*, 1945, 157, 723.

² Astwood, E. B., *J. Am. Med. Assn.*, 1943, 122, 78.

³ Chen, K. K., *Ann. Rev. Physiol.*, 1945, 7, 677.

TABLE I.
Effect of Egg Yolk upon Infection of Mice with Tubercle Bacilli (H37) via Intraperitoneal Route.

Culture H37	Egg yolk suspension	Wt. at weekly intervals after infection				No. dead/ Total inoculated
		Initial	1 wk	2 wk	3 wk	
cc	cc	g	g	g	g	
0.25	0	20.0	20.2	22.6	24.2	0/6
0.25	0.25	20.5	20.7	18.0	Dead	6/6

TABLE II.
Comparison of H37 (Virulent) and H37Ra (Avirulent) Strains Inoculated Via Intravenous Route.

Culture	Egg yolk suspension		Wt. at weekly intervals after inoculation					No. dead/ Total inoculated
			Initial	1 wk	2 wk	3 wk	4 wk	
	cc	cc	g	g	g	g	g	
H37	0.01	0	18.3	21.3	22.2	19.7	19.2	5/6
H37Ra	0.2	0	18.3	21.6	24.2	25.9	26.7	0/6
H37Ra	0.2	0.1	18.3	20.9	23.3	24.6	25.3	0/6

differences in susceptibility among the different strains of mice. In general, albino mice derived from the so-called Swiss strain are somewhat more resistant than the white mice of the Rockefeller Institute strain. On the contrary, a number of other strains such as C57 black, dba, and wild mice (*Mus musculus domesticus*) are markedly more susceptible. The C₃H strain is intermediate between the latter group and the Rockefeller Institute strain.

Albino Swiss mice die within 3 weeks fol-

lowing intravenous injection of 0.05 cc of H37 culture; they survive and exhibit only limited pulmonary lesions when infected with smaller doses. On the other hand, 0.003 cc of culture is sufficient to cause death of dba mice within 3 weeks; moreover, animals of this strain, sacrificed 4-5 weeks after intravenous injection of 0.00003 (corresponding to 0.000005 mg dry weight bacilli), show extensive pulmonary lesions often involving whole lobes.

15738

Alpha Naphthylthiourea (ANTU) in Dogs: Electrophoretic and Cholesterol Studies on Blood Plasma and Pleural Effusion.*

ALFRED CHANUTIN, E. C. GJESSING, AND STEPHAN LUDEWIG. (Introduced by C. P. Richter.)

From the Biochemical Laboratory, University of Virginia, Charlottesville.

Richter¹ recently described a new rat poison, alpha naphthylthiourea (ANTU), which exerts its toxic effect within a few hours by increasing the permeability of the

pulmonary and pleural capillaries. In the dog and rat, the pulmonary edema and pleural effusion cause death. An analysis of the electrophoretic patterns of the serum and effusion fluid in animals poisoned with ANTU provides an approach to the problem of diffusion of proteins across damaged capillary walls.

* This work was done under contract with the Medical Division of the Chemical Warfare Service.

¹ Richter, C. P., *J. Am. Med. Assn.*, 1945, **129**,

TABLE II.
Electrophoretic Analyses of Sera of Dogs 5 and 6 After Multiple Oral Doses of ANTU.
Grams Protein in 100 ml Serum.

Dog No.	Experimental day	ANTU mg/kg	% distribution				Mg N in 100 ml serum			
			Alb.	$\alpha_1 + \alpha_2$	$\beta + \gamma$		Alb.	$\alpha_1 + \alpha_2$	$\beta + \gamma$	
5	1	10	51	14	35		465	130	326	
	3	10								
	4		47	18	35		454	174	331	
	6	15								
	8	25	46	18	36		457	172	352	
	10	40	46	21	33		444	206	321	
	13	60	42	22	36		411	219	358	
	15	75	40	24	36		387	235	355	
	17	75	42	23	35		400	214	337	
	20	150	39	22	39		390	224	392	
	22		48	22	30		515	232	323	
	27		46	22	32		470	230	331	
	31		44	21	35		403	198	318	
6	1	10	56	16	28		385	110	195	
	3	10								
	4		49	25	26		385	198	201	
	6	15								
	8	25	48	20	32		395	164	257	
	10	40	47	21	32		380	168	257	
	13	60	49	20	31		406	161	254	
	15	75	45	21	34		374	174	278	
	17	75	47	21	32		376	172	249	
	20	150	48	19	32		416	164	270	
	24		49	20	31		388	155	240	
	27		50	24	26		427	208	217	
	41		50	21	29		424	180	249	

fluid obtained 15 hours after oral administration. The percentage composition of the serum proteins remained constant throughout the experiment, but the protein concentrations decreased. Appreciable decreases in the milligrams percent of nitrogen occurred in all groups except the γ globulin.

The percentage distribution of the protein groups was almost identical for the serum and effusion fluid at the respective periods. The total nitrogen concentration of the effusion was less than that of the serum, and therefore the content of the various protein groups was proportionately smaller.

Dog 3. (Table I). ANTU was injected intravenously and the chest was tapped hourly. The first effusion fluid was obtained 6 hours after injection at which time blood was also drawn. The dog showed signs of distress shortly afterwards and died $7\frac{1}{2}$ hours after injection. Respiration ceased before the animal could be exsanguinated. Blood was drawn from the heart and approx-

imately 200 ml of effusion fluid were obtained.

The changes in the concentration of the serum fractions were not pronounced. The percentage of the albumin fraction in the pleural effusion was greater than in the serum and the milligram percentage of nitrogen was slightly lower. The nitrogen concentrations of the globulin groups were much lower than those of the serum.

Dog 4. (Table I). This animal was exsanguinated 7 hours after intravenous injection of ANTU. The serum albumin concentration was decreased markedly at death and the globulin groups were only slightly affected. The albumin nitrogen concentration of the effusion fluid was about the same and the concentrations for the globulins were less than those of the serum.

Chronic Poisoning. Two dogs, fed increasingly larger amounts of ANTU in a gelatin capsule, developed a tolerance for doses ordinarily lethal to control animals. The perti-

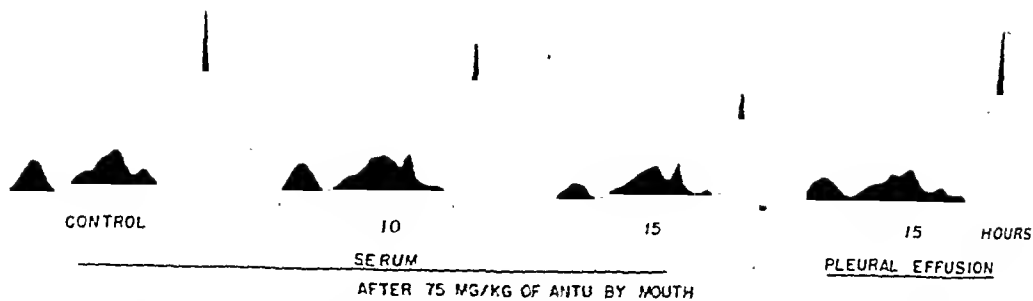


FIG. 1.

Electrophoretic patterns for the serum and pleural effusion of Dog 1 after the administration of ANTU.

(b) the protein and cholesterol concentrations of the serum of dogs which had developed a tolerance to large amounts of this drug.

Methods. Electrophoretic analyses were done in the Tiselius apparatus, using the scanning method of Longworth⁶ and single section cell of 11.0 ml capacity. The serum was diluted 1.5 times with barbiturate buffer, ionic strength 0.1, at pH 8.6. Electrophoresis proceeded for 120 minutes at 15 m. amp. and 200 volts, with a potential gradient of 6.0 volt per cm at 2.0°C.

Cholesterol was determined on plasma by the Schoenheimer-Sperry procedure⁷ with the aid of an Evelyn photoelectric colorimeter with filter No. 660: known amounts of recrystallized cholesterol were used for calibration. The micro-Kjeldahl method was used for total and nonprotein nitrogen.

Results. Protein Distribution. The electrophoretic analyses of 4 dogs given ANTU orally and intravenously are presented in Table I. Owing to the indistinct nature of the electrophoretic patterns of these sera, the proteins are grouped as (1) albumin and α_1 , (2) $\alpha_2 + \alpha_3$, (3) $\beta_1 + \beta_2$, and (4) γ globulins, or a combination of (3) and (4). In group (1), α_1 globulin represents a small proportion of the protein. The data are expressed as percent of total protein and as milligrams percent of nitrogen.

Acute Poisoning. Satisfactory sampling of blood was attained in 2 of 6 dogs which were

given ANTU in gelatin capsules by mouth, and 2 of 4 dogs intravenously injected with ANTU.⁸ In all animals the pulmonary edema was very marked and responsible for their death. Varying amounts of blood-tinged pleural effusion were seen in 9 of the 10 dogs. Usually some respiratory distress was exhibited several hours before death, and great difficulty during the last hour. It was impossible to obtain sufficient serum (10-15 ml) from 6 dogs despite the fact that 100 to 200 ml of blood were obtained when the animal was sacrificed by exsanguination. The blood, of tarry consistency when freshly drawn, was allowed to stand for several hours and was then centrifuged at high speeds. The clot was not firm and did not retract over a period of at least 5 hours. Standing for longer periods yielded a serum which was markedly hemolyzed.

The essential details concerning the 4 satisfactory experimental animals are discussed.

Dog 1. (Table I). Blood was drawn at intervals and the dog sacrificed 15 hours after oral administration of ANTU. The serum albumin concentration decreased markedly and the nitrogen concentration of the $\beta_1 + \beta_2 + \gamma$ globulin group was also lowered.

In the effusion fluid, the albumin concentration was greater than that of the serum at the time of death; the $\alpha_2 + \alpha_3$ group was smaller. The electrophoretic patterns of serum and pleural effusion (Fig. 1) show a striking similarity.

Dog 2. (Table I). The chest was tapped at intervals and the first sample of effusion

⁶ Longworth, L. G., *J. Am. Chem. Soc.*, 1939, **61**, 529.

⁷ Schonheimer, R., and Sperry, W. M., *J. Biol. Chem.*, 1934, **106**, 745.

⁸ Drinker, C. K., *Pulmonary Edema and Inflammation*, Harvard University Press, 1945, 39-43.

TABLE II.
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			Alb.	$\alpha_1 + \alpha_2$	$\beta + \gamma$	Alb.	$\alpha_1 + \alpha_2$	$\beta + \gamma$
5	1	10	51	14	35	465	180	326
	3	10						
	4		47	18	35	454	174	331
	6	15						
	8	25	46	18	36	457	172	352
	10	40	46	21	33	444	206	321
	13	60	42	22	36	411	219	358
	15	75	40	24	36	387	235	355
	17	75	42	23	35	400	214	337
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	22		48	22	30	515	232	323
	27		46	22	32	470	230	331
	31		44	21	35	403	198	318
6	1	10	56	16	28	385	110	195
	3	10						
	4		49	25	26	385	198	201
	6	15						
	8	25	48	20	32	395	164	257
	10	40	47	21	32	380	168	257
	13	60	49	20	31	406	161	254
	15	75	45	21	34	374	174	278
	17	75	47	21	32	376	172	249
	20	150	48	19	32	416	164	270
	24		49	20	31	388	155	240
	27		50	24	26	427	208	217
	41		50	21	29	424	180	249

fluid obtained 15 hours after oral administration. The percentage composition of the serum proteins remained constant throughout the experiment, but the protein concentrations decreased. Appreciable decreases in the milligrams percent of nitrogen occurred in all groups except the γ globulin.

The percentage distribution of the protein groups was almost identical for the serum and effusion fluid at the respective periods. The total nitrogen concentration of the effusion was less than that of the serum, and therefore the content of the various protein groups was proportionately smaller.

Dog 3. (Table I). ANTU was injected intravenously and the chest was tapped hourly. The first effusion fluid was obtained 6 hours after injection at which time blood was also drawn. The dog showed signs of distress shortly afterwards and died 7½ hours after injection. Respiration ceased before the animal could be exsanguinated. Blood was drawn from the heart and approx-

imately 200 ml of effusion fluid were obtained.

The changes in the concentration of the serum fractions were not pronounced. The percentage of the albumin fraction in the pleural effusion was greater than in the serum and the milligram percentage of nitrogen was slightly lower. The nitrogen concentrations of the globulin groups were much lower than those of the serum.

Dog 4. (Table I). This animal was exsanguinated 7 hours after intravenous injection of ANTU. The serum albumin concentration was decreased markedly at death and the globulin groups were only slightly affected. The albumin nitrogen concentration of the effusion fluid was about the same and the concentrations for the globulins were less than those of the serum.

Chronic Poisoning. Two dogs, fed increasingly larger amounts of ANTU in a gelatin capsule, developed a tolerance for doses ordinarily lethal to control animals. The perti-

TABLE III.
Plasma and Pleural Effusion Cholesterol.
Dog given 75 mg/kg of ANTU by mouth.

Hr after ANTU	Total cholesterol, mg %
Plasma.	
Control	95
4	97
6	107
10	91
12	87
13½	79
15	72
Pleural Effusion.	
15	67

nent data for drug administration and for protein distribution in the serum are shown in Table II. There is no typical change in the protein distribution, except a moderate increase in α globulin concentration which can be correlated with the development of tolerance for ANTU.

Plasma Cholesterol. The data for the plasma and effusion cholesterol concentrations after acute poisoning (Table III) indicate a definite decrease in the plasma a few hours before death. The cholesterol concentrations of the plasma and pleural effusion were the same.

Chronic poisoning in 2 dogs was accompanied by a marked increase in plasma cholesterol concentration which probably reflected the "chemical thyroidectomy" caused by ANTU (Table IV). An immediate in-

TABLE IV.
Effect of Chronic ANTU Feeding on Plasma Cholesterol of Dogs.

Day of exper.	ANTU administered mg/kg	Plasma cholesterol mg %	
		Dog 5	Dog 6
1	10	95	118
3	10		
4		153	165
6	15		
8	25	166	176
10	40	194	195
13	60	235	191
15	75	255	215
17	75	287	228
20	150	254	235
22		215	235
24		216	178
27		150	127
31		135	111
41		98	87

crease in cholesterol was seen after the first small doses. The values continued to increase while the ANTU was administered. After its discontinuance, the plasma cholesterol concentration decreased rapidly.

Discussion. In ANTU poisoning, the decrease in the concentration of the protein fractions, particularly in albumin, is apparently a result of the loss of plasma into the lungs and effusion. In the 2 experiments in which the proteins of the pleural effusion were measured before and at death, the albumin concentration increased with time; the data for the globulins are insufficient for drawing any conclusion.

Results of a study of plasma and effusion proteins in a variety of diseases by Luetscher⁹ indicate that the "albumin is present in the same or higher proportion in the effusion, as compared with plasma." Cohn¹⁰ believes that the factor determining diffusion of a protein across a permeable membrane "should be an inverse function of the length of its molecule." This idea is borne out by the data presented by Luetscher,⁹ and by the present experiments.

The effect of ANTU on cholesterol metabolism is striking. The increases noted in chronic experiments are probably associated with the thiourea portion of the molecule and its action on the thyroid. Direct observations on the thyroids were not made.

Summary and Conclusion. Oral and intravenous administration of lethal doses of alpha naphthylthiourea (ANTU) to 4 dogs caused death by affecting the permeability of pulmonary and pleural capillaries, which resulted in marked pulmonary edema and pleural effusion. Electrophoretic and nitrogen determinations of the sera showed consistent decreases in the concentration of albumin several hours before death. The proteins of the pleural effusion showed a higher albumin:globulin ratio than those of the serum. The total plasma cholesterol concentration decreased shortly before death and

⁹ Luetscher, J. A., Jr., *J. Clin. Invest.*, 1941, 20, 99.

¹⁰ Cohn, E. J., *Proc. Am. Philosoph. Soc.*, 1944, 88, 159.

was approximately the same in the effusion.

Dogs fed increasingly greater amounts of ANTU during frequent intervals develop a tolerance for large doses of the drug. An increase in the serum α globulin concentration

appeared to be a characteristic finding in the development of the tolerance. The plasma cholesterol concentration increased markedly during drug administration and diminished when it was discontinued.

15739 P

Folic Acid (Pteroylglutamic Acid)* Studies: Hematologic Remissions in Pernicious Anemia.

ROY R. KRACKE AND WILLIAM H. RISER, JR.

From the Departments of Clinical Pathology and Internal Medicine (Hematology Clinic), The Medical College of Alabama,[†] Birmingham.

Since the *Lactobacillus casei* factor (folic acid) was synthesized in 1945 by Angier and others,¹ many clinical investigators have clearly demonstrated its effectiveness in the treatment of Addisonian pernicious anemia, the anemia of sprue, nutritional macrocytic anemias and the macrocytic anemias of pregnancy. It has likewise been shown that folic acid is quite effective in producing a complete hematologic remission in certain severe macrocytic hyperchromic anemias of infants which are characterized by poor nutrition, a transient histamine fast achlorhydria and a marked megaloblastic hyperplasia of the bone marrow. The treatment of certain human macrocytic anemias with folic acid (pteroylglutamic acid) has been reported by Darby, Jones and Johnson,² Spies and co-workers,³

Moore and co-workers,⁴ Goldsmith,⁵ Doan, Wilson and Wright,⁶ Zuelzer⁷ and others. These extensive studies have been made possible by the chemical identification and synthetic preparation of folic acid (pteroylglutamic acid) in sufficient amounts to permit extensive clinical investigation in several fields. Within the past 12 months we have used synthetic folic acid in treating a great number of different blood dyscrasias. The full report of our investigations will be published in a later communication.

This report deals with the effectiveness of folic acid in the treatment of 4 untreated cases of classical Addisonian pernicious anemia in severe relapse. All of the patients in this group were hospitalized for thorough study and control observations prior to the institution of folic acid therapy. In addition to the usual laboratory procedures for classifying and studying an anemia, sternal marrow aspirations, complete X-ray and fluoroscopic studies of the gastrointestinal tract and gastric analyses were done on each patient. None of these patients exhibited involvement of the central nervous system. A typical hematologic remission is graphically illustrated by the erythrocyte, hemoglobin and reticulocyte response indicated in Chart 1.

* The synthetic folic acid (Folvite) used in this clinical investigation was kindly supplied by Stanton M. Hardy, M.D., Medical Director, Lederle Laboratories, American Cyanamid Company, Pearl River, N.Y.

† The authors wish to express their gratitude to Miss Helen May Holt for her technical assistance in this investigation.

¹ Angier, R. B., and others, *Science*, 1945, **102**, 227.

² Darby, W. J., Jones, E., and Johnson, H. C., *J. A. M. A.*, 1946, **130**, 780.

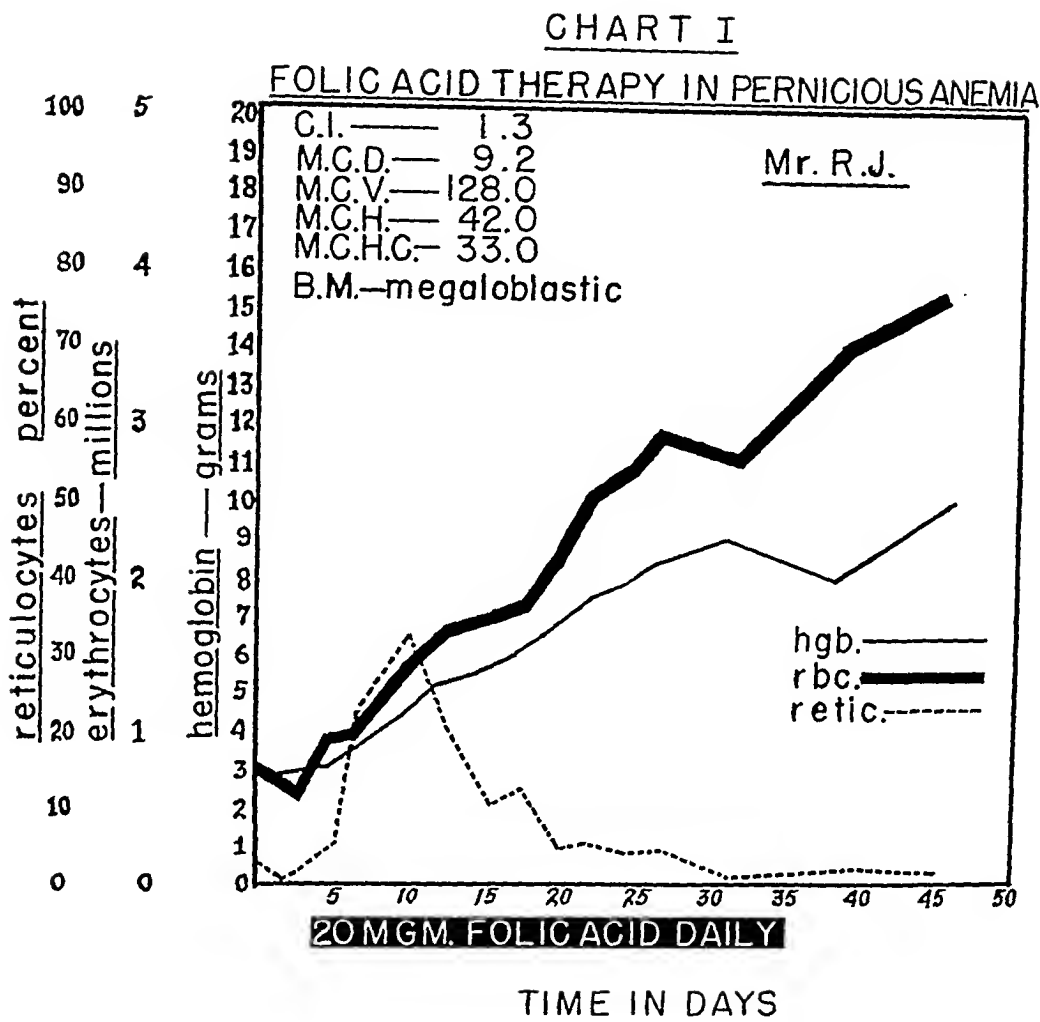
³ Spies, T. D., Vilter, C. F., Koeh, M. B., and Caldwell, M. H., *South. M. J.*, 1945, **38**, 707.

⁴ Moore, C. V., Bierbaum, O. S., Welch, A. D., and Wright, L. D., *J. Lab. and Clin. Med.*, 1945, **30**, 1056.

⁵ Goldsmith, G. A., *J. Lab. and Clin. Med.*, 1946, **31**, 1186.

⁶ Doan, C. A., Wilson, H. E., Jr., and Wright, C. S., *Ohio State M. J.*, 1946, **42**, 139.

⁷ Zuelzer, W. W., *J. A. M. A.*, 1946, **131**, 7.



Similar responses were obtained in 3 other cases.

Case Mr. R. J. The initial erythrocyte count was 770,000 cu mm and hemoglobin was 3 g. This patient was given 20 mg of folic acid (pteroylglutamic acid) daily by mouth, and the hematologic response was prompt. At the present time this patient is in a state of complete hematologic remission (7 months later).

Discussion. Subjective and objective clinical improvement appeared to coincide with the time of maximum reticulocytosis. In all of these cases clinical improvement was rapid and equally as good as is ordinarily observed

in pernicious anemia patients receiving adequate liver extract therapy. In the folic acid-treated group it appears that maximum reticulocytosis is greatest in those cases with erythrocyte levels below one million. Our group of patients has been maintained on 10 mg of folic acid daily by mouth.

It is clearly evident that folic acid (pteroylglutamic acid) will produce a satisfactory hematologic remission in cases of classical Addisonian pernicious anemia, and it appears that these patients can be maintained in remission on a daily oral dose of about 10 mg. None of our patients on this therapy have had or developed central

nervous system disease, so that complete evaluation of folic acid in that respect cannot be made at this time. However, Doan and Moore⁸ independently have recently reported the development and progression of central nervous system disease in pernicious anemia patients while receiving folic acid therapy. Folic acid appears to be equally as effective as liver extract therapy in the treatment of uncomplicated cases of Addisonian pernicious anemia. While folic acid is effective in producing a favorable hema-

⁸ Doan, C. A., and Moore, C. V., personal communication to R. R. K.

tologic remission in cases of pernicious anemia, it is certain that liver extracts contain active material other than this compound, since the potency of such extracts is greater than can be accounted for on the basis of their folic acid content.

Conclusion. 1. Folic acid (pteroylglutamic acid) is as effective as liver extract in correcting the hematologic deficiency in Addisonian pernicious anemia. 2. Further experience is needed before one can evaluate the effectiveness of this factor in preventing development and progression of central nervous system disease.

15740

Effect of Frequency of Hypothalamic Stimulation upon Bladder Response.

BÖRJE UVNÄS. (Introduced by H. W. Magoun.)

From the Departments of Anatomy and Physiology, Northwestern University, Chicago, Ill.

Bladder contraction has been observed by many investigators as the result of the stimulation of the hypothalamus (Karplus and Kreidl,¹ Hess² and others). From their experiments on cats Kabat, Magoun and Ranson³ and Magoun⁴ concluded that a center for the contraction of the bladder is localized in a region just rostral to the hypothalamus and that fibres from this region run caudalward through the lateral part of the hypothalamus. Relaxation of the bladder was only infrequently observed but points giving inhibitory responses were found in different regions of the diencephalon (Kabat *et al.*³) Beattie and Kerr⁵ claimed the existence of centers for the increase of bladder tonus in the anterior hypothalamus and for inhibition

in the posterior hypothalamus and upper mid-brain.

The blood pressure and respiratory responses to hypothalamic stimulation have been shown to be strongly influenced by the frequency of the stimulus (Hare and Geohagan,⁶ Bronk, Pitts and Larrabee;⁷ Berry, McKinley and Hodes⁸). In the present experiments the effect of frequency of hypothalamic stimulation upon bladder response was observed.

Methods. Twenty-four cats under intravenous chloralose anesthesia (50 mg per kg body weight) were used. The hypothalamus was stimulated by a bipolar electrode, made by cementing together 2 lengths of enameled nichrome wire, and oriented with the Horsley-Clarke technic. A thyatron stimulator (Rahm), delivering impulses within a frequency range of 3.5 to 1000 per second, was

¹ Karplus, J. P., and Kreidl, A., *Arch. ges. Physiol.*, 1909, **129**, 138.

² Hess, W. R., *Arch. f. Psychiatr.*, 1936, **104**, 548.

³ Kabat, H., Magoun, H. W., and Ranson, S. W., *J. Comp. Neurol.*, 1936, **63**, 211.

⁴ Magoun, H. W., *Am. J. Physiol.*, 1938, **122**, 530.

⁵ Beattie, J., and Kerr, A. S., *Brain*, 1936, **59**, 302.

⁶ Hare, K., and Geohagan, W. A., *J. Neurophysiol.*, 1941, **4**, 266.

⁷ Bronk, O., Pitts, R. F., and Larrabee, G., *Res. Publ. Nerv. Ment. Dis.*, 1940, **20**, 323.

⁸ Berry, C., McKinley, W., and Hodes, R., *Am. J. Physiol.*, 1942, **135**, 338.

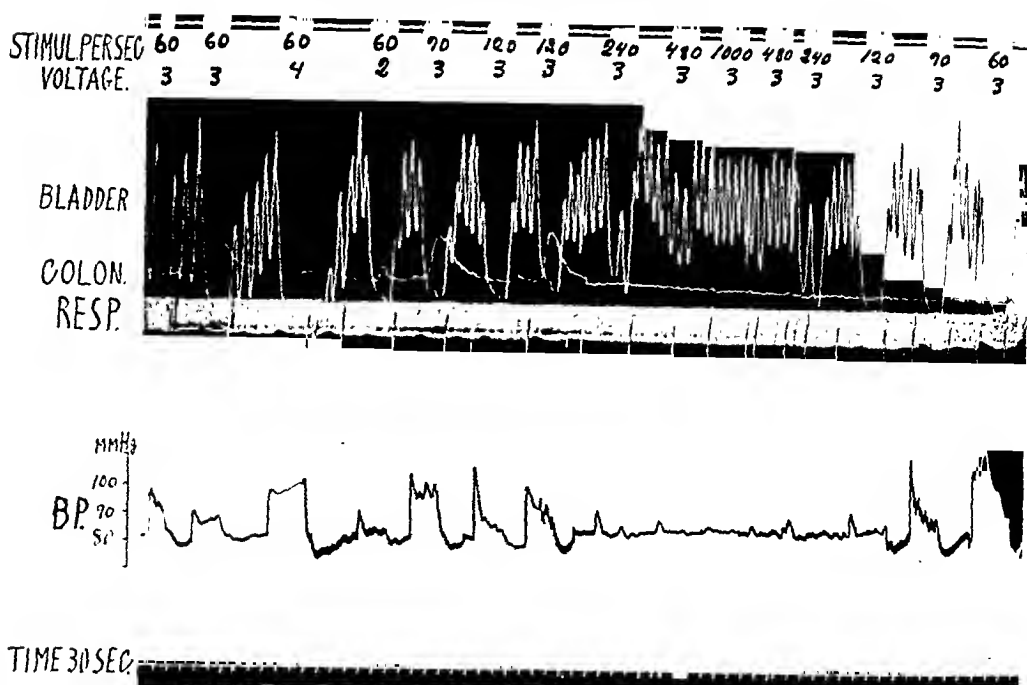


FIG. 1.

Alterations in bladder, colon, blood pressure, and respiration on stimulation of a point in the posterior part of the supraoptic region. Frequency varied.

employed. The pressure of the bladder was recorded by a water manometer connected to a cannula inserted into the bladder through the urethra. In about half the cases the brain was prepared for microscopical examination.

Results. Stimulation of numerous points in the hypothalamus and the preoptic region yielded changes in the intravesical pressure and the peristaltic activity of the bladder. The effects could be either excitatory or inhibitory, the response obtained being strongly influenced by the frequency of the stimulus used. It was an almost regular finding that high frequency stimuli (60/sec. or more), if effective, yielded inhibitory and low frequency stimuli (3.5/sec.), if effective, excitatory responses. The ranges of frequency usually giving inhibitory or excitatory responses are shown in Fig. 1 and 2. As seen, stimuli of a frequency from 15 to 240/sec. yielded a significant inhibition of the bladder. The intravesical pressure as well as the peristaltic activity was depressed. The fre-

quency range giving a pronounced fall of the intravesical pressure and a complete inhibition of the peristalsis in this experiment was observed to be between 30 and 120/sec. At 15/sec. a small initial rise was observed to precede the fall of intravesical pressure and the pressure started to rise before the end of the stimulation. A similar tendency to "escape" was seen at the stimulation with 240 impulses per second. Stimuli of higher frequency were ineffective. The inhibition of the bladder caused by stimuli within the most effective range usually outlasted the stimulation for a considerable time, sometimes for as much as 1-2 minutes or more. Lowering the frequency of the stimuli to 3.5/sec. caused a reversal of the response. A contraction occurred as seen at No. 9 and 10 in Fig. 2. The contractions induced did not persist during the whole period of stimulation, the pressure tending to decline to the initial level after the peak of the contraction was reached. The latent period for the excitatory as well as the inhibitory responses

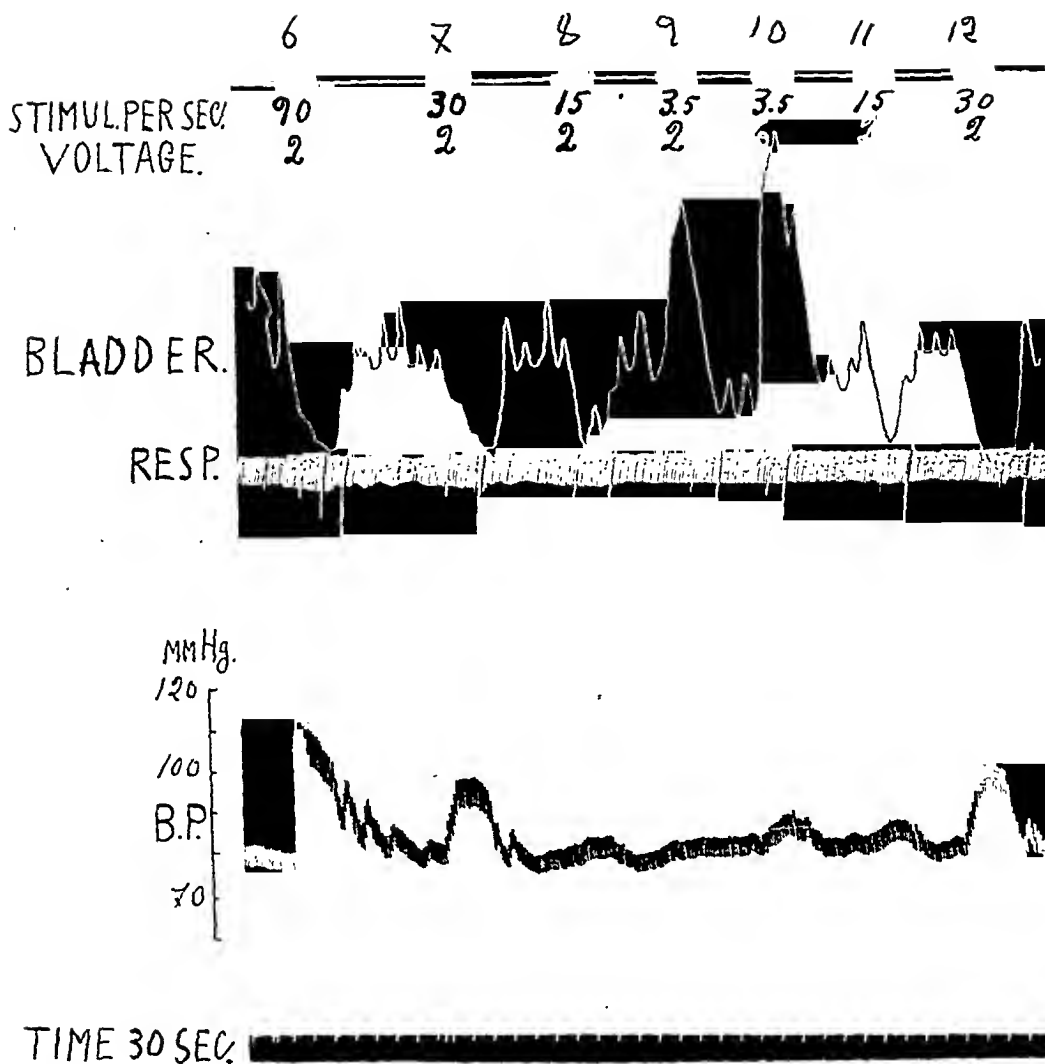


FIG. 2.

Alterations in bladder, blood pressure and respiration on stimulation of a point in the supraoptic region. Frequency varied.

was short, responses being regularly seen to start in a few seconds after the beginning of the stimulus. Fig. 3 illustrates the fact that points yielding bladder contractions will be missed unless stimuli of low frequency are used. As observed by other investigators no correlation was observed between the bladder, blood pressure and respiratory responses.

No definite conclusions can be drawn about the strict localization of the points concerned with bladder activity until a more complete

microscopical investigation is made. From the material at hand points giving bladder contractions were found in the preoptic region as well as in different regions of the hypothalamus. Most of them however were concentrated in the anterior part of the hypothalamus and the posterior part of the preoptic region. Most of the excitatory responses could be reversed to inhibitory ones by increasing the frequency of the stimulus. In addition, numerous points were found

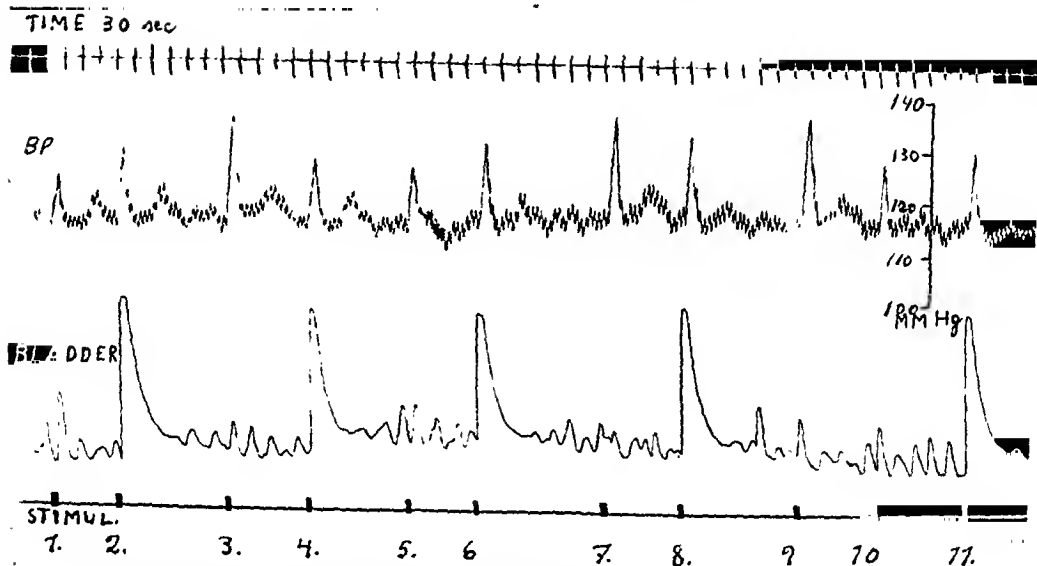


FIG. 3.

Alterations in bladder and blood pressure on stimulation of a point in the supraoptic region. Frequency and intensity of stimulus varied.

No.	Stimul./sec.	Voltage (V)	No.	Stimul./sec.	Voltage (V)
1.	7.5	0.1	3.	60	1.0
2.	"	1.0	5.	60	0.8
4.	"	0.5	7.	60	1.2
6.	"	0.8	9.	120	0.8
8.	"	1.2	10.	60	0.8
11.	"	0.8			

scattered throughout the preoptic region, hypothalamus and upper mid-brain from which only inhibition could be elicited.

Discussion. The experiments have shown that the bladder response to hypothalamic stimulation is strongly influenced by the frequency of the stimulus. High frequency stimuli usually give inhibitory, low frequency stimuli usually give excitatory bladder responses. Most of the excitatory responses could be reversed to inhibitory ones by increasing the frequency of the stimulus. Whether the reversal effects are the result of the activation of different nervous structures or whether the same nervous elements are influenced in a different way by stimuli of different frequency or whether some other explanation is to be found, awaits further determination.

In the study of Kabat *et al.*,³ Magoun⁴ and Wang and Harrison⁹ points giving inhibition of the bladder were only rarely seen, a fact

probably due to the absence of bladder activity in their animals under barbiturate anesthesia. The inability to observe inhibition under these conditions together with the fixed frequency of the stimulus employed (Harvard inductorium), makes it apparent that these investigations did not give a complete picture of the localization of nervous structures concerned with bladder activity. It would seem necessary to reinvestigate this problem using stimuli of varying characteristics.

In their experiments on chloralosed cats Beattie and Kerr⁵ reported that points yielding inhibition of the bladder were only found in the posterior part of the hypothalamus and the upper mid-brain. The present results, showing points giving inhibitory bladder responses scattered throughout the whole hypothalamic area, do not favor the view held by these authors that the hypothalamus can be divided in an anterior excitatory and a posterior inhibitory part.

Summary. The bladder response to hy-

⁹ Wang, S. C., and Harrison, F., *Am. J. Physiol.*, 1939, 125, 2.

pothalamic stimulation is strongly influenced by the frequency of the stimulus used. High frequency stimuli (60/sec. or more) usually cause bladder relaxation, low frequency stimuli (3.5/sec.) usually bladder contraction. A reversal of the bladder response is frequently obtained by changing the fre-

quency of the stimulus.

Points yielding excitation and inhibition of the bladder are found scattered throughout the whole preoptic and hypothalamic area but points giving bladder contraction seem to be concentrated in the rostral parts of the diencephalon.

15741 P

Mitotic Activity in Hypophysis of Pregnant Rat after Injections of Estrogen.

THOMAS E. HUNT.

From the Department of Anatomy, Medical College of Alabama, Birmingham, Alabama.

Division of cells by mitosis increases in the hypophysis of young mature rats in the late estrous phase of the sexual cycle.¹ That this is due to the increase in the level of estrogen is shown by the fact that mitoses can be increased by injections of estrogenic hormones.² Thus, 25 μ g of estradiol benzoate (Progyron-B)* injected into 3-month-old ovariectomized rats 72 and 48 hours before death will result in an average mitotic activity of 22.09 mitoses per square mm of section. During pregnancy, however, even though there is a continued production of estrogenic hormone, mitoses are rare in the hypophysis after the 3rd day.³ Just what level of estrogen occurs during pregnancy is not known, and it seems possible that either the level is not sufficiently high to cause an increased mitotic activity or that there is some substance that inhibits the mitosis-stimulating effect of the estrogen that is produced.

In order to test this point, a group of 22 pregnant animals approximately 3 months of age were injected with 25 μ g of estradiol benzoate, 72 and 48 hours before death. In

addition to these, 3 others that did not receive injections were killed on the 13th day of pregnancy to serve as controls. Of the pregnant animals that received injections of estradiol benzoate, 9 were killed on the 6th, 7th or 8th day of pregnancy and 13 on the 12th, 13th, 14th or 15th day. Of those killed early in pregnancy, 3 animals showed evidence that implantation had occurred but in 2 of these abortion had apparently occurred. Those killed later in pregnancy all had living fetuses.

To determine the mitotic activity, all mitotic figures were counted in a 3-micra coronal section of known area from each hypophysis.

The results are summarized in Table I. Animals receiving injections before implantation of ova (Group 1) have an average of 17.33 mitoses per sq mm. This is not significantly different ($P > .4$) from the results obtained by injecting ovariectomized animals (Group 4),² and, as with the latter group, there is a considerable range in mitotic activity. The animal having the lowest count (3.3 mitoses per sq mm) was the only one that showed normal implantation sites.

Animals injected later in pregnancy and killed from the 12th to the 15th day have an average mitotic activity of 4.35 per sq mm (Group 2). Nine of the 13 animals have consistently low counts (average 1.5), the other 4 falling within the range found for

¹ Hunt, T. E., *Anat. Rec.*, 1942, **82**, 263; *Endocrinology*, 1943, **32**, 334.

² Hunt, T. E., *Anat. Rec.*, in press.

* The estradiol benzoate (Progyron-B) was kindly furnished by the Schering Corporation, Bloomfield, N.J.

³ Hunt, T. E., *Anat. Rec.*, 1943, **85**, 32.

TABLE I.
Mitotic Activity in the Hypophysis of Rats.

Group		No.	Age, avg and range, days	Mitoses per mm ² Mean \pm S.E.
1	Pregnant 6-8 days with estrogen	9	92 (89-93)	17.33 \pm 3.37
2	Pregnant 12-15 days with estrogen	13	94 (83-106)	4.35 \pm 1.41
3	Pregnant without estrogen	3	95 (94-98)	.72 \pm .64
4	Ovariectomized with estrogen	10	83 (76-90)	22.09 \pm 5.46

the injected ovariectomized Group 4. In comparing Groups 2 and 4, the difference is definitely significant statistically ($P. < .01$).

The 3 animals not receiving injections and killed on the 13th day have an average mitotic activity of .72. This is essentially the same as the low mitotic activity found previously in somewhat older animals after the 3rd day of pregnancy³ and is also not significantly different from the average found in ovariectomized animals of the same age.²

The results show that during pregnancy

something is produced that, in most cases, suppresses the mitosis-stimulating effect of estrogen. The fact that the suppression does not occur until after implantation suggests that the placenta produces the inhibiting substance. Since, in some cases, the mitotic activity in the hypophysis is not suppressed, it is necessary to assume either that the inhibiting effect may be overcome or that the cells of the hypophysis are less refractory to mitosis-stimulating substances in some animals.

15742

The Use of a Resistance Wire, Strain Gauge Manometer to Measure Intraarterial Pressure.

EDWARD H. LAMBERT AND EARL H. WOOD.

From the Acceleration Laboratory and Section on Physiology, Mayo Foundation, Rochester, Minn.

For use in measurement of intraarterial pressure in man under ordinary circumstances in the laboratory or clinic, the high frequency, hypodermic manometer developed by Hamilton and his associates¹ has not been excelled in respect to its combination of accuracy and simplicity of operation. In certain unusual situations, however, this manometer is inconvenient to use, because it must be rigidly fixed with respect to the recording camera and records must be made within a few feet of the subject. Attempts to overcome these disadvantages have led to development of means of converting pressure to electrical energy,²⁻⁴ so that arterial blood

pressure might be recorded with more freedom at some distance from the subject by means of a galvanometer. The purpose of this paper is to describe the use of a resistance wire, strain gauge pressure transmitter to accomplish electrical translation in measurement of arterial blood pressure.

The strain gauge pressure transmitter*⁵ consists of a balanced Wheatstone bridge

² Rein, H., *Arch. f. d. ges. Physiol.*, 1940, **243**, 329.

³ Hampel, A., *Arch. f. d. ges. Physiol.*, 1940, **224**, 171.

⁴ Lilly, J. C., *Rev. Scient. Instruments*, 1942, **13**, 34.

* Manufactured by Statham Laboratories, 8222 Beverly Blvd., Los Angeles, Calif.

⁵ Meyer, R. D., *Instruments*, 1946, **10**, No. 3.

¹ Hamilton, W. F., Brewer, G., and Brotman, I., *Am. J. Physiol.*, 1934, **107**, 427.

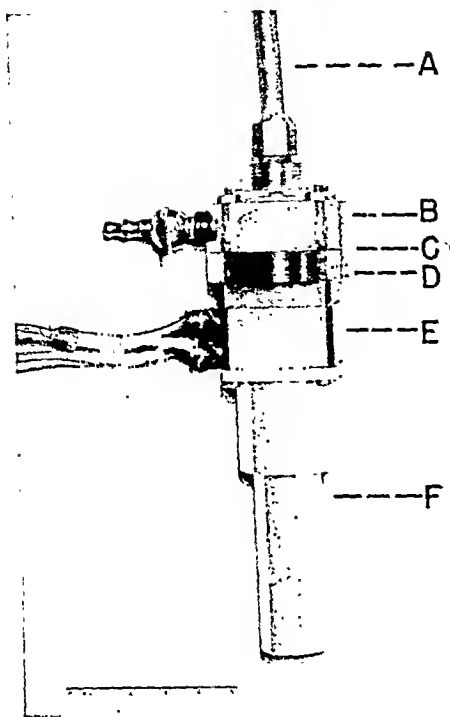


FIG. 1.

Strain gauge pressure transmitter adapted for measurement of arterial blood pressure. A. Lead tube to which needle is attached. B. Lucite chamber filled with anticoagulative solution. Stopcock for fluid reservoir. C. Plastic membrane (Koroseal). D. Lucite chamber filled with oil. E. Case containing strain gauge. Wires lead to battery and galvanometer. F. Holder.

whose 4 elements are made up of strain sensitive wire. The 4 wires are mounted on a cantilever suspension, movement of which increases the strain on one pair of wires and decreases it on the other. Since the resistance of the wires changes with variation in strain, movement of the suspension destroys the balance of the bridge and causes current to flow in the output circuit. The magnitude of the current is directly proportional to the amount of movement of the suspension. The bridge may be powered by dry cells (6 to 20 volts) and the output current measured by means of a microammeter or suitable recording galvanometer. No amplification is necessary. In utilizing this device as a pressure transmitter the cantilever suspension is connected to a bellows or diaphragm. Movement of the bellows or diaphragm caused by applica-

tion of fluid or gas pressure alters the strain on the bridge circuit so that the amount of pressure applied can be determined by measuring the output current.

In order to adapt the pressure transmitter for use in measurement of blood pressure, the housing which surrounds the bellows through which pressure is transmitted to the strain sensitive wires has been replaced by a cylindric lucite chamber (Fig. 1). The latter is filled under vacuum with nonvolatile oil (evacuated hydraulic fluid) and is separated from a second lucite chamber by means of a loose plastic membrane (Koroseal). To the second chamber is attached a lead tube and needle used for arterial puncture and a stopcock which leads to a fluid reservoir. This system is filled with anticoagulative solution consisting of air-free solution of 0.9% sodium chloride containing 20 mg of sodium heparin per liter. Use of oil in the first chamber prevents corrosion of the metal bellows by the action of the anticoagulative solution. Isolation of the first chamber from the second by means of a plastic membrane eliminates the possibility that air bubbles may become lodged in the folds of the bellows whenever the manometer is filled and washed with anticoagulative fluid.

Strain gauge pressure transmitters which have several ranges of sensitivity are manufactured. The gauge which has been used for measurement of arterial blood pressure has a closed circuit output of about 14 microamperes for a pressure equivalent to 100 mm of mercury (bridge resistance 273 ohms) over a total range of ± 780 mm of mercury. With use of this gauge, a lead tube $\frac{1}{8}$ inch (0.3 cm) (inside diameter) by 18 inches

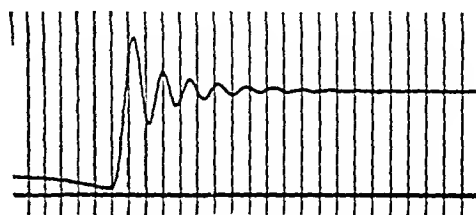


FIG. 2.
Natural frequency of strain gauge manometer with 18-inch lead tube and 19-gauge needle. Time interval is 0.01 second.

TABLE I.
Natural Frequency of Manometers Using Various Strain Gauge Pressure Transmitters.

Strain gauge No.	Full scale range, mm Mercury	Needle* gauge	Natural frequency, cycles per second	
			18-inch lead tube†	No lead tube
PV-107	± 780	19	63	78
		17	80	106
PG-8D-350	± 415	19	29	33
		17	33	50
PG-4D-250	± 208	19	20	25
		17	24	36

* 1¼ inches in length.

† ⅜ inch internal diameter.

(45.7 cm) and a 19-gauge needle 1¼ inches (3.2 cm) long, the natural frequency of the manometer is about 60 cycles per second (Fig. 2) as determined by the method described by Hamilton and his co-workers.² The displacement of fluid into the needle is about 0.5 cu mm when a pressure of 100 mm of mercury is applied.

More sensitive strain gauge pressure transmitters are available, such as those which have a total range of ± 415 mm of mercury or ± 208 mm of mercury, but their fluid displacement is greater and the natural frequency of the manometer using these gauges is considerably lower than that described in preceding sentences (Table I).

The galvanometer which has been used with the pressure transmitter is the Type A manufactured by the Heiland Research Corporation.[†] The natural undamped frequency of this galvanometer is 40 cycles per second. When critically damped, its deflection is within ± 3% of the true response up to a signal frequency of about 35 cycles per second but falls to 50% at about 60 cycles per second. With the galvanometer located 2 meters from the recording camera, the sensitivity of the entire manometer system is such that a deflection of 50 mm is equivalent to a pressure of 100 mm of mercury. The calibration of the system is strictly linear throughout its range and is stable so long as the voltage of the batteries is maintained. Hysteresis is approximately 0.5%. Moderate changes in temperature have only a negligible effect on the system.

† Heiland Research Corporation, 130 East Fifth Avenue, Denver, Colo.

With the exception that recording is done by galvanometer, the techniques and precautions used in measuring pressure with the strain gauge manometer are the same as those employed with the Hamilton manometer.¹ However, because of the relatively large volume of fluid which enters the strain gauge manometer when pressure is applied, special care has been taken to avoid clotting of blood which enters the needle while measurement of blood pressure is being made. The bore of the stainless steel needle is polished by drawing through it a thread impregnated with thick grease containing fine emery dust. Before the needle is used, its inside surface, particularly near the point, is coated very thinly with paraffin. While the needle is in the artery, it is flushed periodically with a very small quantity of sterile anticoagulative solution. With these precautions, pressures have been measured continuously for more than an hour.

The disadvantage of the strain gauge manometer which has been described herein is the low natural frequencies of the manometer and the galvanometer. To determine the limitation which this may impose on use of the strain gauge manometer, the accuracy of this manometer for recording arterial blood pressure has been tested by comparison with a Hamilton manometer which had a natural frequency of 115 cycles per second. Simultaneous recordings obtained by use of the 2 manometers in studies on dogs have demonstrated that the strain gauge manometer accurately records normal peripheral pulses (Fig. 3), but that it may introduce instrumental errors in recording more abrupt cen-

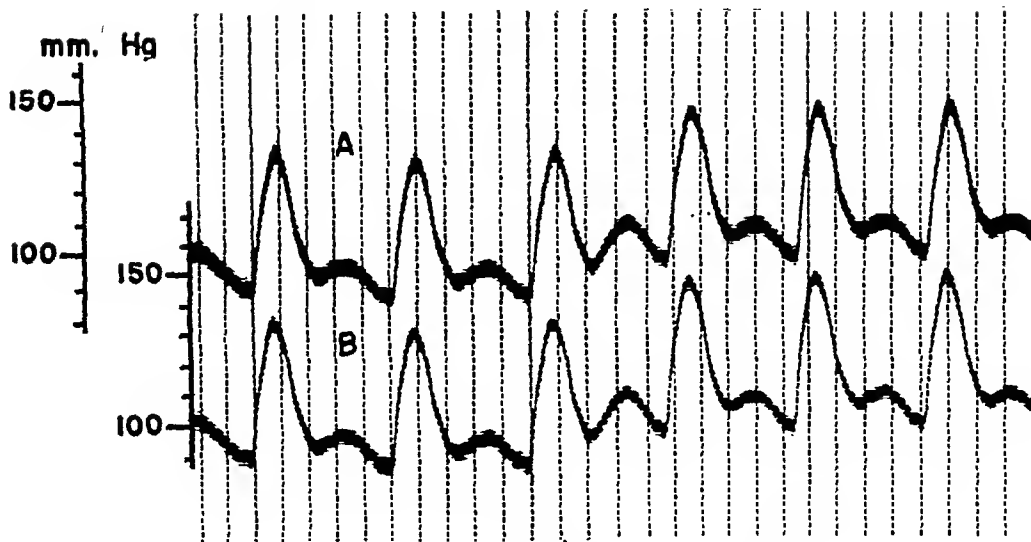


FIG. 3.

Femoral arterial blood pressure of a dog recorded simultaneously by Hamilton manometer (A) and strain gauge manometer (B). Time interval is 0.1 second.

tral pulses as, for example, those of the common carotid artery. For this reason the strain gauge manometer cannot be recommended for the study of arterial blood pressure and pulse wave forms in clinical investigations in which the Hamilton manometer can be employed.

To increase the natural frequency of the strain gauge manometer would require sacrifice of its sensitivity. Because of the limited sensitivity and the low frequency response of recording devices presently available, the use of electronic amplifiers would then be necessary to obtain satisfactory records. Other arrangements of strain sensitive, resistance wire designed for use with amplifiers have been employed in physiological research,⁶ but their suitability for recording arterial blood pressure has not yet been reported.

In its present stage of development, however, the strain gauge manometer which has been described herein is useful for measuring the pressure in peripheral arteries in situations in which electrical translation is necessary. For example, this manometer has been

⁶ Grundfest, Harry, Hay, J. J., and Feitelberg, Sergei, *Science*, 1945, 101, 255.

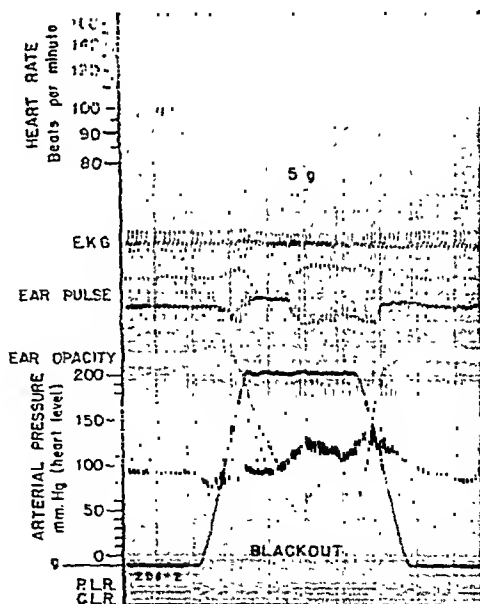


FIG. 4.

Arterial blood pressure and other physiologic variables in man during exposure to centrifugal force (g). Pressure measured in radial artery with wrist at heart level by means of strain gauge manometer. Vertical white lines are spaced at intervals of 5 seconds. P.L.R. and C.L.R. show subject's response to light signals in peripheral and central fields of vision.

useful in determining the changes in arterial blood pressure which occur in man during exposure to centrifugal force on a human centrifuge^{7,8} (Fig. 4). In this case the output current of the strain gauge was carried from the rotating centrifuge through mercury ring contacts and recorded in another room about 60 feet from the subject.

Summary. The use of a resistance wire, strain gauge to convert pressure to electrical energy in making direct measurements of arterial blood pressure is described. The

electrical circuits involved are simple. Recording is by means of a galvanometer without the use of electronic amplifiers. The calibration of the manometer is linear, stable and relatively insensitive to temperature changes. The natural frequencies of the manometer and galvanometer are 60 and 40 cycles per second, respectively.

Addendum. Manometers constructed with strain gauges of a more recent model (P6-15D-250, serial No. 275) have natural frequencies up to 100 cycles per second when fitted with an 18-inch (45.7 cm) lead tube and a 19-gauge needle. Their sensitivity has remained the same as that of previous strain gauge manometers.

⁷ Lambert, E. H., and Wood, E. H., *Fed. Proc.*, 1946, 5, 59.

⁸ Wood, E. H., Lambert, E. H., Baldes, E. J., and Code, C. F., *Fed. Proc.*, 1946, 5, 327.

15743

Sodium Cyanide: Time of Appearance of Signs as a Function of the Rate of Injection.*

ARTHUR A. WARD, JR. (Introduced by W. S. McCulloch.)

From the Department of Psychiatry, University of Illinois College of Medicine, Illinois Neuro-psychiatric Institute, Chicago, Ill.

The effects of sudden intravenous injections of NaCN in animals have been described elsewhere.¹ On the basis of both human and animal studies,² certain conclusions have been drawn as to the probable detoxification rate for this compound based on the assumption that this rate is constant. Because of possible therapeutic applications, it is important to determine in what manner the body responds to slow, constant infusions of dilute solutions of the sodium salt of cyanide, and whether the detoxification rate varies as a function of the rate of injection.

Method. Experiments were carried out

on cats of about 2 kg paralyzed with dihydro-beta-erythroidine hydrobromide[†] (10 mg *i.v.* followed by about 0.1 mg per min. continuously) and maintained on artificial respiration at 25 strokes/min. of 35 cc/stroke. The skull was laid bare and the electrical activity of the cortex obtained with skull electrodes was recorded with a 6-channel, ink writing Grass oscillograph, the EKG being recorded with the same apparatus, using the conventional leads. Changes in the oxygen tension of the cortex were concurrently recorded.³ NaCN was injected continuously[‡] by an accurately calibrated 50 cc syringe driven by a synchronous motor, delivering from 0.033 ml/min. to 0.54 ml/min. By

* The work described in this paper was carried out under a contract between the Medical Division, Chemical Corps, U. S. Army, and the University of Illinois College of Medicine.

¹ McCulloch, W. S., and Wheatley, M. D., *in press*.

² Loevenhart, A. S., Lorenz, W. F., Martin, H. G., and Malone, J. Y., *Arch. Int. Med.*, 1918, 21, 109; Loevenhart, A. S., Malone, J. Y., and Martin, H. G., *J. Pharm. and Exp. Therap.*, 1922, 19, 13.

[†] The author wishes to thank Merek & Co. who kindly supplied this drug.

³ Davis, E. W., McCulloch, W. S., and Roseman, E., *Am. J. Psychiat.*, 1944, 100, 825.

[‡] The author wishes to thank Dr. R. K. Richards of the Abbott Laboratories who loaned to us this apparatus.

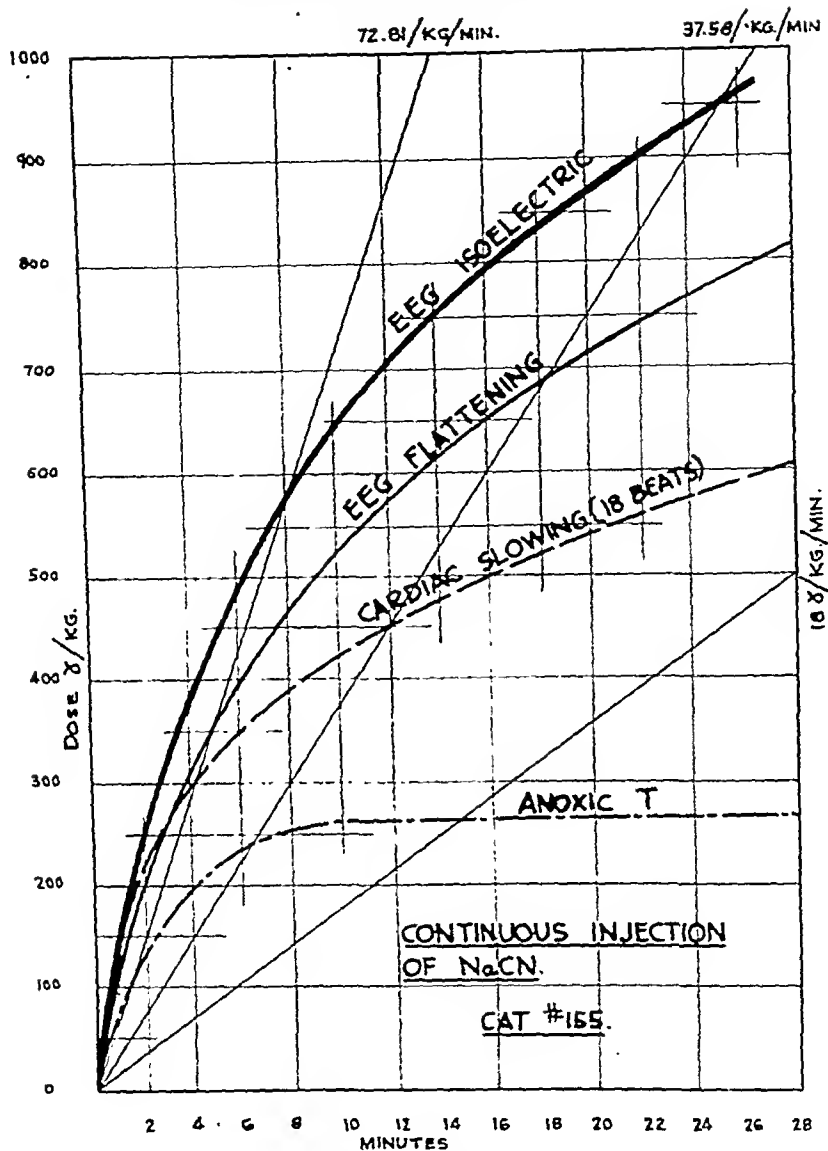


FIG. 1.

Continuous injection of NaCN at the rate of 18, 37, and 72 $\mu\text{g/kg/min}$. (diagonal straight lines). The time of appearance of the various phenomena is plotted against time at these various rates of injection (curved lines).

varying the concentration of NaCN in the solution, rates of injection from 7 $\mu\text{g/kg/min}$. to 100 $\mu\text{g/kg/min}$. could be achieved with accuracy over the entire range.

Results. The effects of slow, constant infusion of NaCN are similar to those which follow sudden injection,¹ the time of onset varying with the rate of injection. One of

the first effects is a slight increase in both the frequency and amplitude of the electrical brain waves which is soon followed by the appearance of large, slow waves, with occasional superimposed bursts of fast, small waves. This is in turn followed by a gradual reduction in both the amplitude and frequency, ending, if the dose is great enough.

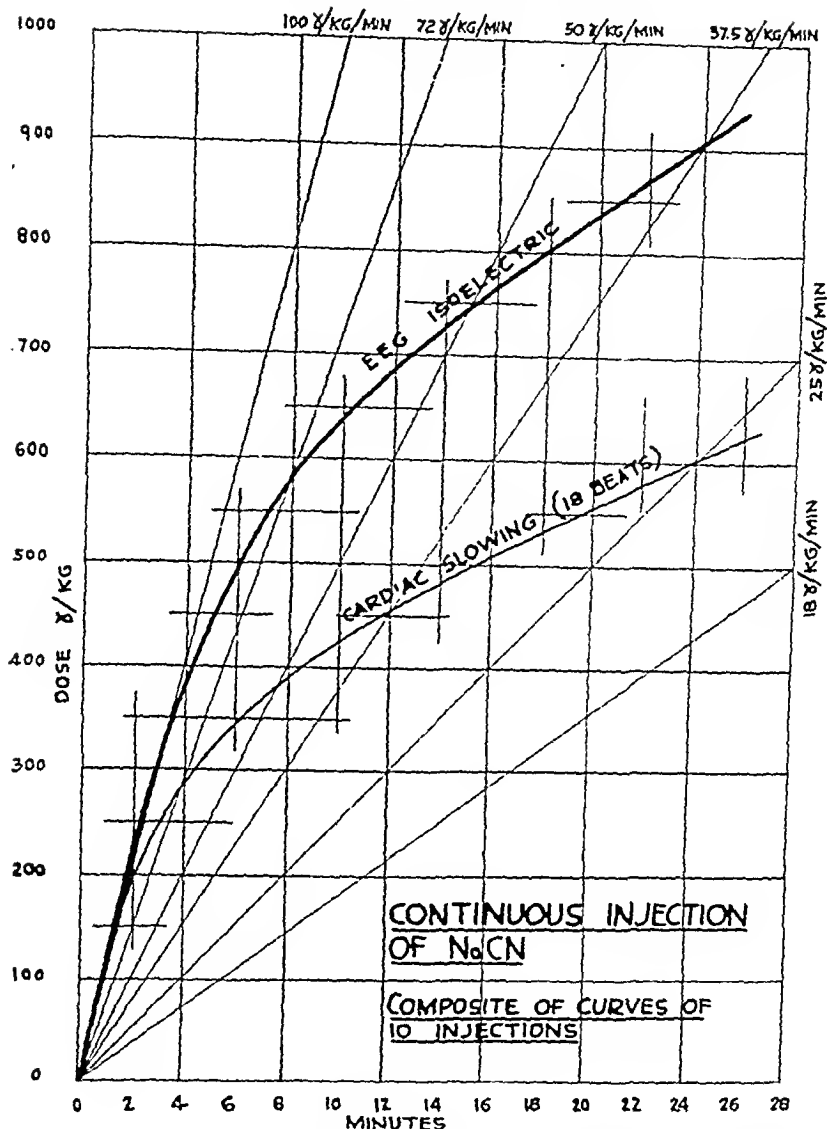


Fig. 2.

Composite curves of 10 experiments. The points of intersection of the curved and diagonal lines indicate particular phenomena occurred at that rate of injection. Dose is expressed in $\mu\text{g}/\text{kg}$.

in an absence of activity. The first cardiac change is an anoxic T-wave which becomes progressively larger and may invert. Slowing of the heart occurs slightly later and increases as the injection continues until the plateau for that dose is reached.

During the recovery from the effects of continuous injection, the electrical activity of the cortex often shows a period of hyper-

activity with episodes of both tonic and clonic types of discharges. This has also been observed following sudden injections.

In contrast to the findings following sudden injection, the oxygen tension fails to show a consistent elevation, even when the EEG changes are most marked.

As an index of the action of NaCN , various clear and consistent phenomena have

been chosen as end-points, namely: onset of anoxic T-wave; cardiac slowing of 18 beats/min.; increase in frequency and also flattening of the electrical activity of the cortex; and onset of isoelectricity of the EEG. The onset of these, abstracted from the continuous records, are plotted on the straight lines representing the rates of injection. The points for each variety of end-point lie on curved lines. The complete curves for one representative experiment (Cat No. 155) are presented (Fig. 1) as well as one composite curve containing the average values for 10 animals (Fig. 2). It will be noted that while the various effects of NaCN occur serially close in time at high rates of injection, at slower rates of injection they are disproportionately spread out in time.

Discussion. It is not surprising that the oxygen tension is inconsistent when both the consumption and the supply are variably decreased.

If detoxification of NaCN in the body proceeded at a constant rate, the plots of the end-points of any variety would fall on a straight line. The observed ascending curves being convex upward, indicate that the rate of detoxification increases with the concentration—as it would if it followed the law of a mass action. The clinical correlate of this concept is that a greater margin of safety obtains at slower rates of injection and for

higher end-points. Thus in Fig. 1 there is an interval of 3 minutes between EEG isoelectricity and EEG flattening when NaCN is injected at 73 $\mu\text{g/kg/min.}$ and an interval of 7 min. at 38 $\mu\text{g/kg/min.}$ At slower rates the interval increases greatly and at 25 $\mu\text{g/kg/min.}$ isoelectricity never occurs.

As the rate of injection is increased so as to approach the injection of the whole dose in 7 seconds, new factors begin to determine the time of appearance of the end-points. These include not only circulation time and time for diffusion into the tissues, but the time for the alteration of the tissue and the time for the development of the particular phenomenon as well as other things at present unknown. Any attempt to extrapolate from the curves for slow injection to the time of appearance of any sign after sudden injection is clearly unwarranted. It is important to note that these other factors determine delays too short to account for the observed curvature.

Summary. NaCN in dilute solution has been injected slowly at constant rates ranging from 7 to 100 $\mu\text{g/kg/min.}$ The time and dose, at which several phenomena are first encountered, plot in ascending curves which are convex upward. This curvature probably indicates that detoxification of NaCN proceeds rapidly at higher concentrations.

15744

Effects of Use and Disuse on Nerve Endings, Neurosomes, and Fiber Types in Skeletal Muscle.*

EBEN J. CAREY, EUGENE HAUSHALTER, LEO C. MASSOPUST, FRANK GAROFALO, JOHN LYNCH, DENIS TABAT, AND ELI SOCOLOFF.

From the Department of Anatomy, Marquette University School of Medicine, Milwaukee, Wisc.

Pathologic neurosomes were first identified in the muscles of man¹ and monkey² during

the early acute changes produced by poliomyelitis, and later in the muscles of rats and

* Aided by a grant from The National Foundation for Infantile Paralysis, Inc., the Baruch Committee on Physical Medicine, and the Medical Department of Hoffmann-La Roche, Inc.

¹ Carey, E. J., Massopust, L. C., Zeit, W., and Haushalter, E., *J. Neuropath. and Exp. Neurol.*, 1944, 3, 121.

² Carey, E. J., *Am. J. Pathol.*, 1944, 20, 961.

VARIOUS RATES OF CYANIDE INJECTION

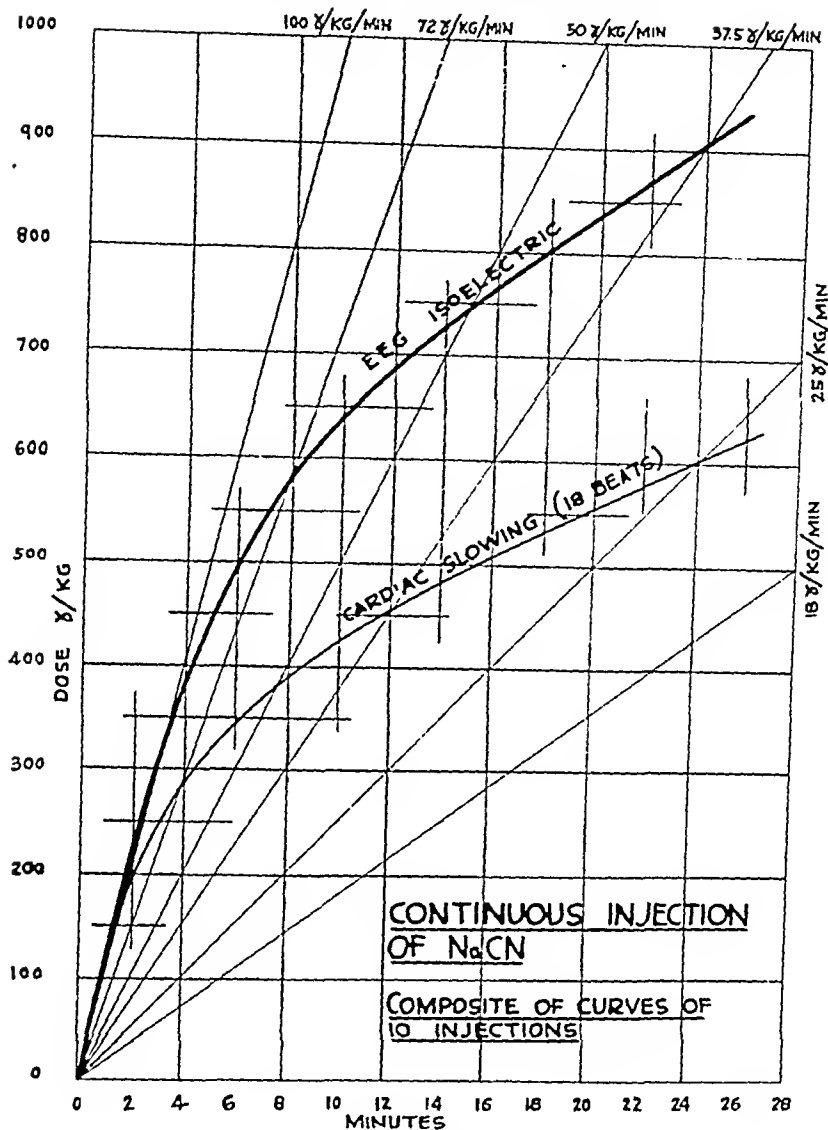


Fig. 2.

Composite curves of 10 experiments. The points of intersection of the curved and diagonal lines indicate particular phenomena occurred at that rate of injection. Dose is expressed in $\mu\text{g/kg}$.

in an absence of activity. The first cardiac change is an anoxic T-wave which becomes progressively larger and may invert. Slowing of the heart occurs slightly later and increases as the injection continues until the plateau for that dose is reached.

During the recovery from the effects of continuous injection, the electrical activity of the cortex often shows a period of hyper-

activity with episodes of both tonic and clonic types of discharges. This has also been observed following sudden injections.

In contrast to the findings following sudden injection, the oxygen tension fails to show a consistent elevation, even when the EEG changes are most marked.

As an index of the action of NaCN, various clear and consistent phenomena have



FIG. 1 TO 3.

Photomicrographs $\times 150$. Normal innervation, Fig. 1; discharge of giant, fusiform neurosomes from end plates 15 days after tenotomy, Fig. 2; and atrophy of nerve endings and muscle 30 days after tenotomy, Fig. 3. whole, teased, gastrocnemius muscle fibers, white rat. Legend: ne, nerve endings; GNS, giant fusiform neurosomes. Gold chloride technic.

chameleons during the onset of shock following hemorrhage,³ heat,⁴ trauma,⁵ chemical action,⁶ and histamine injection.⁷ Suggestive evidence of the probable relation of the periodic secretion from the nerve endings to the granular and agranular muscle fibers, as well as to the clumping effect of DDT⁸ on the neurosomes, had been demonstrated. The discharge of pathologic neurosomes into muscle during the early stages following nerve section⁹ had likewise been shown, as well as the possible relation of the progressive loss of the normal, fine neurosomes to the gradual disappearance of the normal, dark and granular muscle fiber during atrophy. The purpose of this paper is the histologic demonstration of the giant fusiform neurosomes, discharged from nerve endings, retarded in rate of discharge, diffusion, and dissolution during the early stages of muscular atrophy of disuse following tenotomy of the innervated gastrocnemius muscle of the rat.

Methods. Under aseptic surgical technic, the Achilles tendon of the right gastrocnemius muscle was completely severed by transverse section from the calcaneus, and 3 mm of the distal end of the tendon were excised, in 150 white rats (*Mus norvegicus*). The gastrocnemius muscle of the left leg was allowed to remain intact and used as a control of the effects of normal use. At 24-hour intervals the morphologic changes in the right

gastrocnemius muscle were compared with the normally used muscle of the left side in each of 5 rats over a 30-day period. Segments of the control and experimental gastrocnemius muscle and sciatic nerve were simultaneously subjected to the same histologic technics. In 30 additional white rats, 2 were selected at 48-hour intervals following tenotomy. A ligature tied at the cut end of the muscle had at the free end of the ligature suspended weights that varied from 10 to 30 g depending upon the size of the muscle. The living muscle *in situ* was gradually restretched prior to excision and gold impregnation for 10- to 15-minute periods alternating with 5 minutes of rest for 3 to 5 hours.

The method of gold impregnation and teasing of whole muscle fibers, previously described,¹⁻⁹ was found superior to any other neurologic technic for the detection of the structural changes of the nerve endings in muscle. This method was checked against the current popular ones in which silver or methylene blue are used. The muscles were likewise stained with osmic acid, Sudan III, Sudan black, and Scharlach R, as well as with ordinary stains such as hematoxylin and eosine, after fixation with either formalin, Zenker's fluid, or other fixatives. Formalin produced chemical changes and altered critical features of the morphology of the neuromuscular apparatus. No alcoholic dehydrating agent was used in the gold method of teased whole mounts. The teasing of whole muscle fibers was a better technic than that of cutting the muscle into sections for observation of the whole neuromuscular apparatus and the anatomical relationships and changes of the epilemmal axon, hypolemmal axon, granules of Kühne, cross striations, and the granular and agranular muscle fibers. The following experimental observations will be confined to the structure of nerve endings and muscle revealed by the gold technic.

Results. Effect of normal use on nerve and muscle. The normal muscle observed after gold impregnation and in teased whole muscle fibers had narrow, dark, and coarsely granular muscle fibers (Fig. 1) scattered among others of various diameters which were either finely granular or relatively

³ Carey, E. J., Massopust, L. C., Zeit, W., Haushalter, E., and Schmitz, J., *Proc. Soc. Exp. Biol. and Med.*, 1944, **56**, 115.

⁴ Carey, E. J., Massopust, L. C., Haushalter, E., and Zeit, W., *Proc. Soc. Exp. Biol. and Med.*, 1945, **60**, 121; *Am. J. Path.*, 1946, **22**, 175.

⁵ Carey, E. J., Massopust, L. C., Zeit, W., Haushalter, E., and Schmitz, J., *J. Neuropath. and Exp. Neurol.*, 1945, **4**, 134; Carey, E. J., Massopust, L. C., Zeit, W., Haushalter, E., Hamel, J., and Jeub, R., *Am. J. Path.*, 1945, **21**, 935.

⁶ Carey, E. J., *Am. J. Path.*, 1944, **20**, 341.

⁷ Carey, E. J., unpublished observations.

⁸ Carey, E. J., Downer, E. M., Toomey, F. B., and Haushalter, E., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 76.

⁹ Carey, E. J., Massopust, L. C., Haushalter, E., Sweeney, J., Saribalis, C., and Raggio, J., *Am. J. Path.*, 1946, **22**, 1205.



FIG. 4 TO 7.

Photomicrographs. Cross sections. normal, coarsely and finely granular muscle fibers $\times 400$, Fig. 4; giant fusiform neurosomes in teased whole gastrocnemius muscle fibers $\times 600$, Fig. 5 and 6; and cross sections of fiber containing giant fusiform neurosomes, gastrocnemius muscle, white rat, $\times 600$, Fig. 7. Legend: ne, nerve endings; GNS, giant fusiform neurosomes. Gold chloride technic.

agranular and light (Fig. 1 and 4). These dark fibers were designated as hyperchrysophilous and the light ones as hypochrysophilous and achrysophilous. There were multiple gradations of affinity for gold between the extremes. The nerve endings were usually retracted and deeply impregnated with gold (Fig. 1) in the hyperchrysophilous, dark muscle fibers. In the hypochrysophilous fibers the nerve endings were usually expanded (Fig. 1) and had a decreased affinity for gold. The granules of Kühne usually formed a dense rim around the retracted nerve endings whereas there was a quantitative diminution of these granules, to the point of complete depletion, around the extended branches of the expanded nerve endings produced by neuroprotoplasmic streaming. In a differential count of 5000 nerve endings the retracted endings varied in length from 20 to 40 μ and the expanded endings from 40 to 60 μ .

The normal muscle in cross section (Fig. 4) had relatively narrow fibers with coarse granules or neurosomes and the wide fibers contained medium-sized and fine granules. There were variations in the size of the granules, however, in the fibers of different diameters. In other locations in this same muscle, some of the wide fibers were relatively agranular. The axon, nerve endings, granules of Kühne, and granules in the muscle fiber, all had the same reaction to gold. The size and distribution of the granules were assumed to be related to the different degrees in the process of hydrolysis after the neurosomes were discharged into the muscle.

Effect of tenotomy on nerve and muscle. There was a progressive loss of the differential types of muscle fibers following tenotomy (Fig. 2 and 3). The characteristic normal dark type of granular muscle fiber was gradually lost until, on the 30th day (Fig. 3) following tenotomy, it was very infrequently found. There was, likewise, a great depletion (Fig. 3) of the nerve supply. In some places the hypolemmal axons of the nerve endings were completely absent (Fig. 3) and all that remained were clumps of sole plate nuclei. The structural expression of the

dark, coarsely granular muscle fiber (Fig. 1 and 4) was determined by the presence of the attached and normally functioning muscle with its innervation. The nerve endings became uniformly and deeply impregnated with gold and fusiform in shape (Fig. 2) between the 10th and 20th days following tenotomy in the rat. These fusiform nerve endings were moulded by the progressive shrinkage of the muscle fiber due to loss of muscle substance.

On the 15th day (Fig. 2) of disuse atrophy following tenotomy, there could be seen manifestations of various stages in the discharge of giant fusiform neurosomes from the oblong and spindle-shaped nerve endings which were hyperchrysophilous. From the 3rd to the 15th day following tenotomy, in certain places in the muscle, a progressive increase in the number of the giant neurosomes was found. From the 15th to the 30th day following tenotomy, there was a progressive decrease in the number of these giant neurosomes. The discharged giant neurosomes were irregularly scattered in the myoplasm. Some of these giant fusiform neurosomes were uniformly and deeply impregnated with gold. Some were light in the center and dark at the tapering ends. Others were light at the ends and dark in the center. Still others were very faintly impregnated with gold, and their contained granules were arranged in cross striations undergoing progressive alignment with the cross striations of the muscle fiber.

These fusiform neurosomes varied from 10 to 275 μ in length and from 4 to 60 μ through the widest transverse diameter. In some places there were small oblong and fusiform neurosomes similar, in morphology and staining reaction to gold, to those observed during the early stages after nerve section in denervated muscle.⁹ The large fusiform neurosomes produced a streamlining effect (Fig. 5 and 6) on the cross striations of muscle during the process of migration from the nerve ending and dispersion in the myoplasm of the muscle fiber. Some of these neurosomes in transverse sections were immediately under the sarcolemma whereas oth-

insertion, appear to be necessary for the normal rate of discharge of granules from the motor end plates. This secretory process from the nerve endings may be slowed down by disuse of the innervated gastrocnemius muscle following tenotomy. By tenotomy, the normal stretch or tension of the muscle is destroyed. The lax muscle fibers released from one attachment are analogous to the broken strings of a violin. The detached strings are incapable of normal vibratory response because of loss of tone or tune. The rate of discharge of neurogenic substance into the muscle appears to depend upon the reciprocal interaction between nerve and muscle. The normal mechanical tension of the attached muscle fibers appears to determine the normal periodical flow of neurogenic substances into the muscle. The normally attached muscle fiber appears to act like an alternate pressure and suction chamber upon the nerve ending. This nerve ending appears to be a biological jet valve or ejector of the neurogenic secretion. Under normal conditions the rate of discharge, diffusion, and disappearance of neurosomes is excessively rapid. The release of normal muscle stretch by tenotomy decreases the demand, and slows down the rate resulting in accumulation of the neurogenic discharge into the abnormally flaccid muscle fibers.

Evidence now at hand supports the statement that the changes of fatty metamorphosis, Zenker's hyaline degeneration, the pathogenesis of dystrophy and of poliomyelitic muscle, are closely related to the pathology of the neuromuscular apparatus and alterations in the secretion of neurosomes into muscle. In the past, many of the fine physiologic neurosomes have been identified variously as the interstitial granules of Kölliker,¹⁹ the J and Q granules of Holm-

gren²⁰ and the liposomes of Albrecht²¹ and Bell.²²

Evidence¹⁻⁷ had been accumulated and presented that the full fractional contraction, the relative relaxation, and the onset of contraction of functional activity, all were correlated with the types of nerve endings and muscle fibers. The dark, granular muscle fibers were proportionately increased in number by magnesium sulphate⁹ and by agents that inhibited cholinesterase in its hydrolytic action on acetylcholine, such as thiamin chloride, atropine, curare, prostigmine, and ergotmine.²³ In high concentrations (0.25 to 0.5%) these chemicals injected into the living muscle increase proportionately by 25 to 50% the number of the dark granular muscle fibers.²³ On this incomplete evidence it is assumed that some of the granules in the dark muscle fibers are composed, at least in part, of acetylcholine.

The evidence presented in this paper supports the principle of the *Double Dependence* of nerve and muscle proposed by Young.¹⁰ He stated that muscle receives its stimulation from nerve and exercises constraint against other muscles or outside forces. Muscle will atrophy if given too little direction from above, as after total denervation or isolation of lower from upper neurones; it will also atrophy if it is left relaxed by tenotomy and, therefore, cannot contract against resistance. We have observed, also, that disuse of the relaxed and attached gastrocnemius muscle, in the rat, by fixation of the whole limb in a cast produces changes in the neuromuscular apparatus similar to the effects of tenotomy.²⁴

Summary. The limited experimental evidence presented in this paper tends to support the following statements:

The normally used and innervated gastrocnemius muscle of the white rat is characterized histologically by dark, coarsely granular and light, finely granular and

¹⁹ Kölliker, A., *Z. f. Wissensch. Zool.*, Leipzig, 1857, 8, 311.

²⁰ Holmgren, E., *Anat. Anz.*, 1907, 31, 609; *Arch. f. mikr. Anat.*, 1907-08, 71, 165; *Ibid.*, 1910, 75, 240; *Anat. Anz.*, 1913, 44, 225; *Nervenz.*, 1913, 14-15, 277.

²¹ Albrecht, E., *Verhandl. d. deutsch. path. Gesellsch.*, 1903, 6, 63.

²² Bell, E. T., *Anat. Rec.*, 1910, 4, 199; *Internat. Monatschr. f. Anat. u. Physiol.*, 1911, 28, 297; *J. Path. and Bact.*, 1912-13, 17, 147.

²³ Carey, E. J., unpublished observations.

²⁴ Carey, E. J., unpublished observations.

ers occupied the center (Fig. 7) of the muscle fibers. During disuse atrophy there was a progressive loss of the normal dark muscle fiber and of the normal coarse granules (Fig. 7) of the neurosomes. The granules were either very fine and dispersed, or aggregated into the giant fusiform neurosomes. In cross sections (Fig. 7) the giant neurosomes were either uniformly impregnated deeply with gold, or they had a light center or a rim of granules faintly reacting to gold.

The gastrocnemius muscle lost 30 to 50% of its weight, compared to the normal control, between 21 to 30 days after the tenotomy. There were individual variations in the rate of atrophy and in the morphologic changes of the neuromuscular apparatus and fibers of the gastrocnemius muscles. These variations were found not only in muscles from different animals examined after the same time interval following tenotomy, but also in different fibers in the same muscle. It was necessary, therefore, to survey great numbers of muscle fibers to detect the statistical trend of the morphologic changes.

The reestablishment of partial muscle stretch in some fibers of the muscle by regenerative attachment of the tendon to the subcutaneous tissue was a variable to be taken into consideration in evaluating the results. In some of the rats in our series the tendon of the muscle was freshly cut at 7-day intervals to eliminate the effects of partial reestablishment of stretch by union of tendon to subcutaneous tissue. The loss of normal muscle stretch determined the discharge of giant neurosomes. This was indicated by the total absence of the giant neurosomes in the series of 30 living muscles experimentally restretched *in situ* before excision and gold impregnation. Two muscles were restretched at 48-hour intervals until the 30th day after tenotomy.

Discussion. The basic mechanism in the physiology of use, increased use by exercise, and disuse, resulting, respectively, in muscle maintenance of health, hypertrophy, and atrophy of disuse and disease, is unknown. Young¹⁰ stated that "the theories of the

causes and nature of muscular atrophy are numerous but none is conclusive." This conclusion is held likewise by Carlson and Johnson.¹¹ The parts of the living body increase and decrease in size in proportion to the functional demand or use, but the essential underlying cause of these changes has remained an elusive one.

The hyperexcitability manifested by fibrillations may be prevented by quinidine, but Solandt and Magladery¹² observed that the *denervated muscle continues to shrink in size*. The slow, incoordinate activity of fibrillation, therefore, appears not to be the cause of the muscular atrophy. It has been demonstrated by Gutmann and Gutmann,¹³ Hines,¹⁴ Eccles,¹⁵ and Solandt,¹⁶ that the volume of denervated muscle may be fairly well maintained for a certain period by appropriate electrical exercises. Evidently, periodic optimum tension of traction and contraction (work) of muscles anchored to their attachments is necessary for muscle maintenance during health. Suggestive evidence was demonstrated previously that an optimum periodic tension of differential growth is necessary for the genesis of both smooth and skeletal muscle.¹⁷ Evidence was also demonstrated that anatomic and experimental dampers to the lateral expansion of the stretched muscle fibers produces a replacement of muscle by fibrous tissue.¹⁸

The normal tension, or stretch, and work of the intact muscle attached to origin and

11 Carlson, A. J., and Johnson, V., *The Machinery of the Body*, University of Chicago Press, Chicago, 1937, 620 pp.

12 Solandt, D. Y., and Magladery, J. W., *Brain*, 1940, **63**, 255.

13 Gutmann, E., and Gutmann, L., *Lancet*, 1942, **1**, 169.

14 Hines, H. M., *J. A. M. A.*, 1942, **120**, 315; Hines, H. M., Thomson, J. D., and Lazere, B., *Arch. Phys. Therap.*, 1943, **24**, 69.

15 Eccles, J. C., *J. Physiol.*, 1944, **103**, 253.

16 Solandt, D. Y., de Lury, D. B., and Hunter, J., *Arch. Neurol. Psychiat.*, 1943, **49**, 802.

17 Carey, E. J., *J. Gen. Physiol.*, 1920, **2**, 357; *J. Gen. Physiol.*, 1920, **3**, 61; *Am. J. Anat.*, 1921, **29**, 341; *J. Morphol.*, 1922, **37**, 1.

18 Carey, E. J., *Am. J. Anat.*, 1936, **59**, 89.

10 Young, J. Z., *Lancet*, 1946, **2**, 109.

has been initiated in our laboratories, with results which indicate that the infection prevails among them.

There are two methods which may be employed for the detection of ornithotic infections in birds: one involves the inoculation of mice with the suspected avian spleen, liver and kidney tissue emulsions; the other method involves the serological detection of infection with the complement-fixation technic. That the latter technic is accurate is affirmed by the extensive data presented by Bedson,⁴ and by Meyer *et al.*⁵ Francis and Gordon⁶ increased the specificity of the antigen by preparing it from the chorio-allantoic fluid of the infected chick embryo.

Methods. Antigen. A standard strain of ornithosis virus (P-4), obtained from Dr. J. E. Smadel, was propagated in the chorio-allantoic fluid of 7-day-old chick embryos. After 5 days further incubation at 35°C, the extra embryonic fluids were collected from the eggs and this material was then inactivated at 56°C/30 minutes. The agent was sedimented in an angle head centrifuge at 6000 r.p.m./1 hour after which the sediment was resuspended in saline to 1/10 its original volume. It was then titrated with a known potent antiserum and its complement fixing titer was set at 2 units. This antigen was prepared by us while at the 8th Service Command Laboratory, Fort Sam Houston, Texas, and was subsequently preserved in the lyophilized state at refrigerator temperature until used in this problem.

Serum. The blood specimens were collected by heart puncture and after storage overnight in the refrigerator, the serum was separated and inactivated at 56°C/30 minutes. Specimens of serum were collected from the following species of birds: Laughing gull (*Larus atricilla* L.), Royal tern (*Sterna maxima* B.), Least tern (*Sterna antillarum* L.), Common tern (*Sterna hirundo* L.), Skimmer (*Rynchops nigra* L.), Willet (*Catotrophus*

scnipalmatus G.), Gull billed tern (*Gelochelidon nilotica* L.), Glossy ibis (*Plegadis autumnalis* L.), Reddish egret (*Dichro-manassa refulgens*), Brown pelican (*Pelecanus occidentalis* L.), Sooty tern (*Sterna fuscatus*), and Sanderling (*Calidris leucophaca* P.).

Test. The inactivated serum was diluted in saline serially from 1:5 to 1:160 in 0.2 cc amounts, 2 units of antigen, and 2 units of complement was added, and this mixture was incubated at 37°C/1 hour. The amboceptor and 2% sheep red blood cells were then added and further incubated at 37°C/30 minutes. At the end of this period the results were recorded, providing the serum, antigen and complement controls were satisfactory.

Results. It may be noted in Table I that 40% or more of the laughing gull, skimmers and willets showed complement-fixing antibodies for ornithosis in their blood serum. There was a lower incidence of antibodies in the blood serums of the terns and sanderlings, although the total numbers of the species were smaller. The majority of serum titers were in 1:5 and 1:10 dilutions; however, some went as high as 1:80 and 1:320 dilution. Of 165 birds of all species examined, 61 (36%) showed evidence of complement-fixing antibodies for ornithosis in the blood serum.

This serological evidence must necessarily be confirmed by actually demonstrating the agent in organs of naturally infected birds. Thus far, a virulent ornithosis-like agent has been isolated in mice from the pooled livers and spleens of 2 willets. This agent (W-ornithosis strain) is lethal for mice by intracerebral and intraperitoneal routes; in either case, nests of elementary bodies can be observed in the mononuclear cellular exudate of the spleen and meninges. The agent kills 7-day-old chick embryos in 4 to 5 days, in which case the chorio-allantoic fluid contains numerous minute elementary bodies. An antigen was prepared from the chorio-allantoic fluid, as described for the P-4 psittacosis antigen above, and it fixed complement with psittacosis serum, lymphogranuloma venereum serum, and its homologous rabbit serum. This latter serum also fixed

⁴ Bedson, S. P., *Brit. J. Exp. Path.*, 1936, **17**, 109.

⁵ Meyer, K. F., and Eddie, B., *J. Inf. Dis.*, 1939, **65**, 225.

⁶ Francis, R. D., and Gordon, F. B., *Proc. Soc. Exp. Biol. and Med.*, 1945, **59**, 270.

agranular muscle fibers. The nerve endings are usually retracted in the dark, coarsely granular fiber and expanded in the light, agranular fiber, as revealed by gold impregnation of teased whole muscle fibers. The atrophy of disuse following tenotomy of the gastrocnemius muscle with nerve supply intact, is accompanied, during the first month, by the progressive loss of the narrow and dark, coarsely granular muscle fiber, and by a depletion of its innervation. The dark, granular muscle fiber is determined by the reciprocal interaction of the normally intact and attached muscle fiber with its normally functioning innervation. During the process of atrophy of disuse after tenotomy, small and giant fusiform neurosomes are discharged from the altered nerve endings. It is assumed that these giant fusiform neurosomes

are the product of a retardation in the rate of the discharge; diffusion, and disappearance by hydrolysis, following tenotomy and disuse atrophy of the innervated gastrocnemius muscle. There appears to be a parallelism between the atrophy by disuse of tenotomized muscle and the loss of the normal discharge of neurosomes from the altered and progressively depleted innervation of the muscle. One factor in the atrophy of disuse of muscle appeared, therefore, to be the substantial loss of the discharge of neurosomes into muscle as well as the quantitative decrease of the myoplasm. The giant fusiform neurosomes that appear during the early period following tenotomy disappear when the living muscle *in situ* is adequately restretched prior to excision and gold impregnation.

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Ornithosis in Sea-Shore Birds.*

MORRIS POLLARD. (Introduced by Ludwik Anigstein.)

From the University of Texas, Galveston, Texas.

Psittacosis-like infections have been reported in a large variety of wild and domesticated birds including parrots, finches, pigeons, chickens and turkeys.¹ Because of the wide prevalence of psittacosis-like infection among many species of wild and domesticated birds, Meyer proposed that the more general term "ornithosis" be employed in referring to nonpsittacine infections. It has not yet been found in partridges, quail or wild ducks.² Among the sea birds, the Ful-

mar petrel has been incriminated as the source of an epidemic of psittacosis in human inhabitants of the Faroe Islands.³ Many of the avian species in which this disease is present ordinarily do not manifest clinical symptoms of the disease but carry the virus in "silent" fashion. This dormant infection of birds appears to be provoked to activity by conditions associated with crowding, malnourishment, or other effects of commercial aviary mismanagement. The disease might also be regarded as a natural population controlling factor, remaining inapparent until overpopulation and undernourishment permit the infection to become activated and aggravated in virulence.

Psittacosis (ornithosis) is an important public health problem; so it is important that the natural or potential reservoirs of the disease be known. A study of the incidence of ornithotic infections among sea shore birds

* Sincere appreciation is expressed to Drs. R. W. Strandmann and C. U. Dernehl for their assistance in the capture and identification of the birds used in this study.

¹ Meyer, K. F., Psittacosis and Ornithosis, in *Diseases of Poultry*, The Collegiate Press, Inc., Ames, Iowa, 1944.

² Eddie, B., and Francis, T., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1942, **50**, 291.

³ Rasmussen, R. F., *Zentralbl. f. Bakt.*, I Abt., Orig., 1938, **143**, 89.

In previous communications submitted from this laboratory¹⁰⁻¹² a method was described for preparing specific diagnostic antigens for certain rickettsial and neurotropic viral diseases. The adaptation by Koprowski and Cox^{13,14} of Colorado tick fever virus to the brain tissue of the laboratory mouse provided means of obtaining large amounts of infectious material for the preparation of complement-fixing antigen. The present work deals with the application of the above cited method in the preparation of complement-fixing antigen for the diagnosis of Colorado tick fever.

Materials and Methods. Virus Strains. Three strains of Colorado tick fever virus—the Florio (F1), the Baker (B), and the Condon (C)—originally obtained from Dr. Lloyd Florio*,⁴ and adapted to the mouse brain in this laboratory,^{13,14} were used for the preparation of the complement-fixing antigen. The F1 strain had undergone 30 hamster² and 35 mouse brain passages.¹³ The B strain had been carried through 5 hamster⁴ and 10 mouse brain passages,¹⁴ while the C strain was adapted directly from the serum of a naturally infected human case to the brain of the dilute brown agouti (dba) mouse,¹⁴ following which it was carried through 10 brain-to-brain passages in Swiss albino mice.

Stable virus preparations were readily obtained by preparing 5 or 10% infected brain suspensions in a 50-50 mixture of normal

rabbit serum-saline (pH 7.4) and storing in the dry ice chest.

Antigens. Three groups of 300, 21-day-old Swiss albino mice were injected intracerebrally with 0.03 ml of a 1:50 dilution of infectious mouse brain suspension of the F1, B and C strains of virus, respectively. Ten percent normal rabbit serum in saline was used as diluent. Four days later the animals in all 3 groups showed nervous symptoms but relatively little paralysis. The brains were removed aseptically from all mice of each group and antigens were prepared by lyophilization followed by benzene extraction, as previously reported by De-Boer and Cox.^{11,12} Prior to processing, each lot of infected mouse brain suspension was tested for infectivity by intracerebral inoculation in 21-28-day-old Swiss albino mice. LD₅₀ titers ranging from 10⁻⁶ to approximately 10⁻⁷ were obtained.

Immune serum. The F1 strain was used for the production of all immune serum by hyperimmunizing mice. Swiss albino mice, approximately 2 months old, were injected intraperitoneally with 0.5 ml of a 1:50 dilution of infected mouse brain suspension in distilled water. Thereafter, the mice were reinjected at weekly intervals with 0.5 ml of a 10% brain suspension until tests conducted with serum obtained from trial bleedings indicated that a satisfactory antibody titer was secured. A total of 10 injections was given before the mice were exsanguinated and their sera used for the tests reported herein. In addition several serum samples from clinically diagnosed human cases of Colorado tick fever which occurred in Colorado, obtained through the courtesy of Drs. Lloyd Florio and H. L. Morency,[†] were subjected to the tests.

Complement-fixation Tests. Complement-fixation tests were carried out identically to those reported previously.¹² Antigens were titrated for antigenic activity in the presence of various dilutions of homologous immune sera. The dilution of antigen giving the highest titer for the immune serum was used

* Pollard, M., Livesay, H. R., Wilson, D. J., and Woodland, J. C., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61**, 396.

¹⁰ Wolfe, D. M., Van der Scheer, J., Clancy, C. F., and Cox, H. R., *J. Bact.*, 1946, **51**, 247.

¹¹ De Boer, C. J., and Cox, H. R., *J. Bact.*, 1946, **51**, 613.

¹² De Boer, C. J., and Cox, H. R., *J. Immunol.*, 1947, **55**, 193.

¹³ Koprowski, H., and Cox, H. R., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 320.

¹⁴ Koprowski, H., and Cox, H. R., in manuscript. Presented before the American Society of Tropical Medicine, Miami, Florida, November 5, 1946.

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					1:5	1:10	1:20	1:40	1:80	1:320
Laughing gull	60	3	24	40	10	8	1	2	2	1
Willet	37	3	17	45.4	7	6	4			
Skimmer	15	0	8	53	4	2	2			
Sanderling	8	1	4		4					
Royal tern	15	0	1	6			1			
Least tern	8	0	2			1		1		
Common tern	6	0	3		1	11				
Gull billed tern	6	0	1	16		1				
Ring billed gull	1	0	0							
Glossy ibis	1	0	1						1	
Brown pelican	4	1	0							
Sooty tern	1	0	0							
Reddish egret	3	3	0							
	165	11	61	36	26	20	8	3	3	1

complement with lymphogranuloma venereum antigen and with psittacosis antigen.

The significance of finding evidence of ornithosis in sea-shore birds serves to add to the spectrum of information regarding the distribution of this disease in nature. While it may not be a great hazard to the human population, since these sea-shore birds are not generally confined in close association with man, nevertheless, this disease appears to exist in a multitude of avian species. Under proper circumstances these infected birds

might contribute directly, or through other birds, to the morbidity rate of human ornithosis.

Summary. Serological evidence of ornithosis-like infections among several species of sea-shore birds is presented. Over 40% of the serums of sea gulls, willets, and skimmers which were examined showed complement-fixing antibodies for ornithosis. One strain of an ornithosis-like agent has been isolated from the willet species.

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Specific Complement-Fixing Diagnostic Antigens for Colorado Tick Fever.

CARL J. DE BOER, LAWRENCE J. KUNZ, HILARY KOPROWSKI, AND HERALD R. COX.

From the Section of Viral and Rickettsial Research, Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

In the past Colorado tick fever^{1,2} was believed to be a mild form of Rocky Mountain spotted fever.³ However, largely due to the work of Florio and his colleagues,^{4,5} it is now

known that Colorado tick fever is not a rickettsial infection but a viral disease,⁶ presumably tick-borne,⁶ which apparently is quite distinct from Rocky Mountain spotted fever^{1,3,7} and dengue fever^{8,9} although resembling the latter both clinically and hematologically.

¹ Becker, P. E., *Colorado Med.*, 1930, **27**, 36, 87.

² Toomey, N., *Ann. Int. Med.*, 1931-32, **5**, 585.

³ Florio, L., Mudge, E. R., and Stewart, M. O., *Ann. Int. Med.*, 1946, **25**, 466.

⁴ Florio, L., Stewart, M. O., and Mudge, E. R., *J. Exp. Med.*, 1944, **80**, 165.

⁵ Florio, L., Stewart, M. O., and Mudge, E. R., *J. Exp. Med.*, 1946, **83**, 1.

⁶ Topping, N. H., Callyford, J. S., and Davis, G. E., *Publ. Health Rep.*, 1940, **55**, 2224.

⁷ Shaffer, F. C., *Colorado Med.*, 1935, **32**, 226.

⁸ Florio, L., Hammon, W. McD., Laurent, A., and Stewart, M. O., *J. Exp. Med.*, 1946, **83**, 295.

In previous communications submitted from this laboratory¹⁰⁻¹² a method was described for preparing specific diagnostic antigens for certain rickettsial and neurotropic viral diseases. The adaptation by Koprowski and Cox^{13,14} of Colorado tick fever virus to the brain tissue of the laboratory mouse provided means of obtaining large amounts of infectious material for the preparation of complement-fixing antigen. The present work deals with the application of the above cited method in the preparation of complement-fixing antigen for the diagnosis of Colorado tick fever.

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The significance of finding evidence of ornithosis in sea-shore birds serves to add to the spectrum of information regarding the distribution of this disease in nature. While it may not be a great hazard to the human population, since these sea-shore birds are not generally confined in close association with man, nevertheless, this disease appears to exist in a multitude of avian species. Under proper circumstances these infected birds

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Summary. Serological evidence of ornithosis-like infections among several species of sea-shore birds is presented. Over 40% of the serums of sea gulls, willets, and skimmers which were examined showed complement-fixing antibodies for ornithosis. One strain of an ornithosis-like agent has been isolated from the willet species.

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Specific Complement-Fixing Diagnostic Antigens for Colorado Tick Fever.

CARL J. DE BOER, LAWRENCE J. KUNZ, HILARY KOPROWSKI, AND HERALD R. COX.

From the Section of Viral and Rickettsial Research, Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

In the past Colorado tick fever^{1,2} was believed to be a mild form of Rocky Mountain spotted fever.³ However, largely due to the work of Florio and his colleagues,^{4,5} it is now

known that Colorado tick fever is not a rickettsial infection but a viral disease,⁶ presumably tick-borne,⁶ which apparently is quite distinct from Rocky Mountain spotted fever^{1,3,7} and dengue fever^{8,9} although resembling the latter both clinically and hematologically.

¹ Becker, F. E., *Colorado Med.*, 1930, **27**, 36, 87.

² Toomey, N., *Ann. Int. Med.*, 1931-32, **3**, 583.

³ Florio, L., Mudge, E. R., and Stewart, M. O., *Ann. Int. Med.*, 1946, **25**, 466.

⁴ Florio, L., Stewart, M. O., and Mudge, E. R., *J. Exp. Med.*, 1944, **80**, 165.

⁵ Florio, L., Stewart, M. O., and Mudge, E. R., *J. Exp. Med.*, 1946, **83**, 1.

⁶ Topping, N. H., Cullyford, J. S., and Davis, G. E., *Publ. Health Rep.*, 1940, **55**, 2224.

⁷ Shaffer, F. C., *Colorado Med.*, 1935, **32**, 226.

⁸ Florio, L., Hammon, W. McD., Laurent, A., and Stewart, M. O., *J. Exp. Med.*, 1946, **83**, 295.

TABLE II.
Complement-fixation Tests with Colorado Tick Fever Immune Serum and Various Viral and Rickettsial Antigens.

Antigen	CTF* immune mouse serum										Controls		
	Serum dilutions										Serum dilutions		
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:2	1:4	1:8	1:2	1:4	1:8
Western E.E.	0	0	0	0	0	0	0	0	0	0	0	0	0
Eastern E.E.	0	0	0	0	0	0	0	0	0	0	0	0	0
St. Louis	0	0	0	0	0	0	0	0	0	0	0	0	0
Japanese B	±	0	0	0	0	0	0	0	0	0	0	0	0
Rabies	0	0	0	0	0	0	0	0	0	0	0	0	0
Murine typhus	0	0	0	0	0	0	0	0	0	0	0	0	0
RMSF†	0	0	0	0	0	0	0	0	0	0	0	0	0
CTF*—F†	4	4	4	4	4	2	0	0	0	0	0	0	0
CTF*—B	4	4	4	4	4	2	0	0	0	0	0	0	0
CTF*—C	4	4	4	4	4	1	0	0	0	0	0	0	0
American Q	0	0	0	0	0	0	0	0	0	0	0	0	0

* CTF = Colorado tick fever. †RMSF = Rocky Mountain spotted fever.

TABLE III.
Complement-fixation Tests with Colorado Tick Fever Human Sera.

Serum	Blood taken* months	Antigen	Human sera										Controls				Hemolytic system		
			Serum dilutions										Serum dilutions					Antigen	Red blood cells
			1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:1	1:2	1:4	1:8						
No. 1	33	CTF—F†	±	0	0	0	0	0	0	0	0	0	0	0	0	4	4		
No. 2	2½	CTF—F†	4	4	4	4	±	0	0	0	0	0	4	2	0	0	4	0	
No. 3	2½	CTF—F†	4	4	4	4	±	0	0	0	0	0	4	4	0	0	4	0	
No. 4	42	CTF—F†	±	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	
No. 5	7	CTF—F†	4	4	4	4	1	0	0	0	0	0	0	0	0	0	4	0	
		RMSF	±	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	
		Kolmer	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	
No. 6	34	CTF—F†	4	4	4	4	1	0	0	0	0	0	0	0	0	0	4	0	
		CTF—B	4	4	4	4	0	0	0	0	0	0	0	0	0	0	4	0	
		CTF—C	4	4	4	4	0	0	0	0	0	0	0	0	0	0	4	0	
		RMSF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	
		Kolmer	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	

* Counting from dry when diagnosis of Colorado tick fever was made.

TABLE I. Titration of Colorado Tick Fever Antigen.

Antigen	Dilution	Immune mouse serum						Serum dilutions					Controls			Red blood cells	Hemolytic system
		Serum dilutions						1:4					Antigen				
		1:4	1:8	1:16	1:32	1:64	1:128	1:256	0	0	0	0	0	0	0		
F1	1:1	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	
	1:2	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	
	1:3	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	
	1:4	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	
	1:8	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	
B	1:1	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	
	1:2	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	
	1:3	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	
	1:4	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	
	1:8	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	
C	1:1	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	
	1:2	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	
	1:3	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	
	1:4	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	
	1:8	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	

4 = complete fixation of complement. 3, 2, 1 = proportionately lesser fixation. + = trace, 0 = no fixation.

4 = complete fixation of complement. 3, 2, 1 = proportionately lesser fixation. + = trace. 0 = no fixation.

in subsequent complement-fixation tests. Although this may require more frequent re-checking of the titer of an antigen, it is of value in obtaining the optimal titers of unknown sera. Complement was always titrated in the presence of antigen thus diluted, increasing the amount of 1:30 dilution of complement by 0.025 ml instead of 0.05 ml, thereby obtaining a more precise and sensitive test.

Neutralization Test. Equal volumes of undiluted test serum and aliquots of serial 10-fold dilutions of mouse brain suspension infected with either F1 or C strains of Colorado tick fever virus were mixed, incubated in a water bath at 37°C for 2 hours, and inoculated into 21-28-day-old Swiss albino mice by the intracerebral route. Normal human serum was used as control.

Experimental. Titration of Colorado Tick Fever Antigens. Table I shows the results obtained when the antigens prepared from F1, B and C strains were titrated in the presence of an immune mouse serum prepared with F1 strain. It is apparent that all 3 antigens gave approximately the same degree of fixation of complement with the F1 serum.

In numerous additional tests it was determined that the benzene extracted antigens prepared from each of the 3 strains of Colorado tick fever virus consistently gave negative results in the presence of highly positive Wassermann human sera. These results thus confirm and extend the observations previously reported by DeBoer and Cox^{11,12} in that benzene-extracted antigens are highly specific and do not give false positive reactions in the presence of markedly positive syphilitic sera.

Cross-fixation Tests with Colorado Tick Fever Immune Serum and Heterologous Antigens. To check the antigenic identity of the virus, Colorado tick fever immune mouse serum was tested against a number of viral and rickettsial antigens by the complement-fixation reaction. The Eastern (EEE) and Western (WEE) equine encephalomyelitis antigens were derived from infected chick embryos; the murine (endemic) typhus,

TABLE II.
Complement-fixation Tests with Colorado Tick Fever Immune Serum and Various Viral and Rickettsial Antigens.

Antigen	CTF* immune mouse serum									
	Serum dilutions					Controls				
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	Serum dilutions		Hemolytic system
								1:2	1:4	
Western E.E.	0	0	0	0	0	0	0	0	0	0
Eastern E.E.	0	0	0	0	0	0	0	0	0	0
St. Louis	0	0	0	0	0	0	0	0	0	0
Japanese B	±	0	0	0	0	0	0	0	0	0
Rabies	0	0	0	0	0	0	0	0	0	0
Marine typhus	0	0	0	0	0	0	0	0	0	0
RMSF†	0	0	0	0	0	0	0	0	0	0
CTF*—F1	4	4	4	4	4	2—	0	0	0	0
CTF*—B	4	4	4	4	4	2	0	0	0	0
CTF*—C	4	4	4	4	4	1	0	0	0	0
American Q	0	0	0	0	0	0	0	0	0	0

* CTF = Colorado tick fever. † RMSF = Rocky Mountain spotted fever.

TABLE III.
Complement-fixation Tests with Colorado Tick Fever Human Sera.

Serum	Blood taken* months	Antigen	Human sera										Controls					Hemolytic system
			Serum dilutions										Antigen	Red blood cells	Hemolytic system			
			1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:1	1:2	1:4				1:8		
No. 1	33	CTF—F1	±	0	0	0	0	0	0	0	0	0	0	0	0	4	4	0
No. 2	2½	CTF—F1	4	4	4	4	±	0	0	0	0	0	4	2	0	0	4	0
No. 3	2½	CTF—F1	4	4	4	4	0	0	0	0	0	0	4	4	0	0	4	0
No. 4	42	CTF—F1	±	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0
No. 5	7	CTF—F1	4	4	4	1	0	0	0	0	0	0	0	0	0	0	4	0
		RMSF	±	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0
		Kolmer	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0
No. 6	34	CTF—F1	4	4	4	1	0	0	0	0	0	0	0	0	0	0	4	0
		CTF—B	4	4	4	0	0	0	0	0	0	0	0	0	0	0	4	0
		CTF—C	4	4	4	0	0	0	0	0	0	0	0	0	0	0	4	0
		RMSF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0
		Kolmer	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0

* Counting from day when diagnosis of Colorado tick fever was made.

Rocky Mountain spotted fever (RMSF) and American Q fever antigens were prepared from infected yolk sacs; while the St. Louis and Japanese B encephalitis and rabies antigens originated from infected mouse brains.[‡] The antigens fixed complement in the presence of their homologous antisera in the following dilutions: EEE 1:64; WEE 1:64; murine typhus 1:128; RMSF 1:16; American Q 1:1024; St. Louis 1:64; Japanese B 1:64, and rabies 1:128.

The results obtained in testing the above antigens in the presence of a Colorado tick fever immune mouse serum are shown in Table II. No fixation of complement was obtained with any of the heterologous antigens, although all 3 Colorado tick fever antigens showed good fixation titers.

Complement-fixation Tests with Convalescent Sera. Table III summarizes the results of complement-fixation tests with Colorado tick fever antigen and human sera obtained at different intervals after the onset of the disease.

Serum No. 1 was taken from a person who had suffered a natural infection 33 months previously. Sera No. 2 and No. 3 were taken 2½ months after experimentally induced infections and were stored in the frozen state for 42 months. Serum No. 4 was from the same person as No. 3 but taken 42 months after the original infection and 30 months after another injection of virulent material. Sera No. 5 and No. 6 were from persons who had had natural infections 7 and 34 months prior to bleeding, respectively. These sera were tested for complement-fixing antibodies in the presence of Colorado tick fever, Rocky Mountain spotted fever and Kolmer Wassermann antigens.

The results shown in Table III indicate that sera No. 1 and No. 4 taken 33 and 42 months respectively after an attack of the disease, demonstrated only traces of complement-fixing antibodies in the presence of Colorado tick fever antigen. Sera No. 2 and

No. 3 taken 2½ months after diagnosis of illness, and No. 5 and No. 6 taken 7 and 34 months, respectively, apparently showed significant antibody titers. The specificity of the test is again demonstrated by the fact that sera No. 5 and No. 6 showed fixation only with Colorado tick fever antigen and not with Rocky Mountain spotted fever, nor Kolmer Wassermann antigens. In addition, it is seen that serum No. 5 fixed complement equally well in the presence of Colorado tick fever antigens Fl, B and C. This latter observation would seem to indicate that these 3 strains of Colorado tick fever are very similar antigenically, if not identical.

Comparison of Complement-fixing and Neutralizing Antibodies in Human Convalescent Sera. Table IV shows the results obtained in testing Colorado tick fever convalescent sera for their complement-fixing and neutralizing antibody titers. Since the serum samples were taken at various intervals from infected human cases, this test furnishes additional information regarding the time of appearance of complement-fixing or neutralizing antibodies and the length of time they persist during the convalescent period.

The results of the neutralization tests were in close agreement with those of the complement-fixation tests in all cases where the sera were not anticomplementary. Sera No. 2 and No. 3 were anticomplementary in dilution 1:1 and 1:2 to 2+ and 4+, respectively, which may account for the relatively higher complement-fixing titers (see Tables III and IV).

There is also close correlation in the results obtained with the 2 types of tests performed on sera of patients Ca, Og, Mo and Wi. For instance, the Ca serum, obtained 2 days after the diagnosis of Colorado tick fever, was devoid of all complement-fixing or neutralizing power. On the other hand, the Og serum taken on the 9th day after the disease was diagnosed, showed some complement-fixing and neutralizing capacity. Mo and Wi sera had definite complement-fixing power although their neutralizing titers were low. It may be added, that the results of

[‡] All antigens, with the exception of American Q fever, were prepared by benzene extraction procedures. The American Q fever antigen was an ether extracted-washed rickettsial body preparation.

TABLE IV.
Comparison of Complement-fixation and Neutralization Tests with Colorado Tick Fever Human Convalescent Sera.

Comparison of complement-fixation and neutralization tests with combined																		
Complement-fixation tests†										Neutralization tests‡								Calculated Protective index§
Serum	Blood* taken	Antigen	Serum dilutions						Mortality ratio of mice inoculated with virus dilutions									
			1:1	1:2	1:4	1:8	1:16	1:32	10-1	10-2	10-3	10-4	10-5	10-6	10-7	10-8	LD ₅₀ titer	
No. 1	33 mos.	F	±	0	0	0	0	0	0	6/6	6/6	6/6	6/6	4/5	0/5	1/5	10-5.50	4
No. 2	2½ "	F	4	4	4	4	±	±	0	6/6	6/6	3/5	4/5	0/6	0/5		10-1.10	112
No. 3	2½ "	F	4	4	4	4	±	0	0	6/6	6/6	5/6	3/6	2/6	0/6	0/6	10-1.15	100
No. 4	42 "	F	±	0	0	0	0	0	0	6/6	6/6	6/6	3/6	0/5	0/5		10-1.00	141
No. 5	7 "	F	4	4	4	4	1	0	0	6/6	3/6	0/5	0/5				10-2.00	14,130
No. 6	34 "	F	4	4	4	4	1	0	0	6/6	4/6	1/6	1/6	5/6	1/6	0/6	10-2.05	5,012
Ca	2 days	F	2-	0	0	0	0	0	0	6/6	6/6	6/6	6/6	0/6	0/6		10-5.50	4
Og	9 "	F	3	2	0	0	0	0	0	6/6	6/6	6/6	6/6	0/6	0/6		10-1.50	45
Ma	14 "	F	4	4	4	4	4	3	±	6/6	5/6	1/6	0/6				10-1.50	45
B		B	4	4	4	2	0	0	0									
C		C	4	4	4	1	0	0	0									
Wi	18 "	F	4	4	4	2	0	0	0	6/6	4/6	2/6	1/6				10-1.00	35
B		B	4	3	0	0	0	0	0									
C		C	4	3	0	0	0	0	0									
Normal control																		
Su	2nd febrile rise	F	0	0	0	0	0	0	0	6/6	6/6	6/6	3/6	0/6	1/6		10-0.35	0
B	22 days	B	4	4	4	4	0	0	0	6/6	6/6	6/6	6/6	5/6	0/6	0/6	10-0.10	0**
C		C	4	4	4	4	0	0	0	6/6	6/6	6/6	4/6	0/4			10-5.25	14**
B	76 "	B	4	4	4	3	0	0	0	6/6	5/6	1/4	0/6	1/6			10-3.10	1,000**
C		C	4	4	4	4	0	0	0									
Kolmer		Kolmer	0	0	0	0	0	0	0									

* Counting from day when diagnosis of Colorado tick fever was made.

† Complement-fixation tests made by G.J.D. and L.J.K.

‡ Neutralization tests made by H.K.

§ Based on difference in LD₅₀ titer from normal control serum.

|| Induced experimental infection.

** Sera No. 2 and No. 3 were anticomplementary in dilutions 1:1 and 1:2 to 2+ and 4+ respectively.

*** Based on difference between convalescent and acute phase sera.

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The results of the neutralization tests were in close agreement with those of the complement-fixation tests in all cases where the sera were not anticomplementary. Sera No. 2 and No. 3 were anticomplementary in dilution 1:1 and 1:2 to 2+ and 4+, respectively, which may account for the relatively higher complement-fixing titers (see Tables III and IV).

There is also close correlation in the results obtained with the 2 types of tests performed on sera of patients Ca, Og, Mo and Wi. For instance, the Ca serum, obtained 2 days after the diagnosis of Colorado tick fever, was devoid of all complement-fixing or neutralizing power. On the other hand, the Og serum taken on the 9th day after the disease was diagnosed, showed some complement-fixing and neutralizing capacity. Mo and Wi sera had definite complement-fixing power although their neutralizing titers were low. It may be added, that the results of

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TABLE IV.
 Comparison of Complement-fixation and Neutralization Tests with Colorado Tick Fever Human Convalescent Sera.

Complement-fixation test†										Neutralization test‡										Calculated Protective index§
Sera dilutions										Mortality ratio of mice inoculated with virus dilutions										
Serum	Blood* taken	Antigen	1:1	1:2	1:4	1:8	1:16	1:32		10-1	10-2	10-3	10-4	10-5	10-6	10-7	10-8	LD ₅₀ titer		
No. 1	33 mos.	F1	±	0	0	0	0	0	0	6/6	6/6	6/6	6/6	4/5	0/5	1/5		10-5.50		
No. 2 1/2 "	2 1/2 "	F1	4	4	4	4	4	±	0	6/6	6/6	3/5	4/5	0/6	0/5			10-1.10		
No. 3 1/2 "	2 1/2 "	F1	4	4	4	4	4	0	0	6/6	6/6	5/6	3/6	2/6	0/6			10-1.15		
No. 4 1/2 "	4 1/2 "	F1	±	0	0	0	0	0	0	6/6	6/6	6/6	3/6	0/5	0/5			10-1.00		
No. 5 "	7 "	F1	4	4	4	1	0	0	0	6/6	3/6	0/5	0/5					10-2.00		
No. 6 "	34 "	F1	4	4	4	1	0	0	0	6/6	4/6	1/6	1/6					10-2.45		
Cu	2 days	F1	2-	0	0	0	0	0	0	6/6	6/6	6/6	6/6	5/6	1/6	0/6		10-5.50		
Og	9 "	F1	3	2	0	0	0	0	0	6/6	6/6	6/6	6/6	0/6	0/6			10-1.50		
Mo	14 "	F1		4	4	4	3	±	0	6/6	5/6	1/6	1/6	0/6				10-1.50		
		B		4	4	2	0	0	0											
		C		4	4	1	0	0	0											
Wt	18 "	F1		4	4	2	0	0	0	6/6	4/6	2/6	1/6					10-1.00		
		B		4	3	0	0	0	0											
		C		4	3	0	0	0	0											
Normal control																				
8u	2nd febrile rise	F1	0	0	0	0	0	0	0	6/6	6/6	3/6	0/6	1/6				10-0.15		
	22 days	B	4	4	4	4	0	0	0	6/6	6/6	6/6	5/6	0/6				10-0.40		
		C	4	4	4	4	0	0	0	6/6	6/6	4/6	0/4					10-5.25		
76	"	B	4	4	4	3	0	0	0	6/6	5/6	1/4	0/6	3/6				10 3.10		
		C	4	4	4	4	0	0	0											
		Kolmer	0	0	0	0	0	0	0											

* Counting from day when diagnosis of Colorado tick fever was made.
† Complement-fixation test.
‡ Neutralization test.
§ Calculated from day when diagnosis of Colorado tick fever was made.

* Counting from day when diagnosis of Colorado tick fever was made.

† Complement-fixation tests made by C.F.D. and L.J.K.

‡ Neutralization tests made by H.K.

 § Based on difference in LD₅₀ titer from normal control serum.

¶ Sera No. 2 and No. 3 were anticomplementary in dilutions 1:1 and 1:2 to 2+ and 4+ respectively.

** Based on difference between convalescent and acute phase sera.

complement-fixation on the above sera were in close agreement regardless of whether the F1, B or C antigens were used in the test.

The results of the tests with Sn sera are perhaps more illustrative than the above cited instances. Serum samples were obtained from the same individual at various intervals during and after illness. It may be observed that the Sn serum drawn during the second febrile rise was free from complement-fixing, as well as neutralizing antibodies. On the other hand, serum taken 22 days later showed positive complement-fixation in 1:8 dilution but neutralized only 14 LD₅₀ doses of virus in mice. The complement-fixing antibody titer showed no significant difference between the 22nd and 76th days after diagnosis, but the neutralizing index of the serum increased from 14 to 1,000 during the same period. Again, the results of complement-fixation with the 3 samples of Sn serum were the same regardless of whether B or C antigens were employed in performing the test.

Summary and Conclusions. Specific diagnostic complement-fixing antigens for Colorado tick fever have been prepared from in-

fect mouse brains. The benzene-extracted antigens employed gave no false positive reactions in the presence of highly positive human syphilitic sera. Cross fixation tests between Colorado tick fever immune serum and the heterologous antigens of viral and rickettsial origin indicate that Colorado tick fever is a distinct entity and the virus is not related to any of the other infectious agents tested. These results confirm those obtained with the mouse neutralization test previously reported.^{13,14} Close correlation was obtained in the complement-fixation and mouse neutralization tests with human convalescent sera. The complement-fixing and neutralizing antibodies apparently appear in the blood of humans at about the 9th to 14th day after diagnosis of illness and may remain demonstrable as long as 34 months later.

The complement-fixation test, as well as the mouse neutralization test, may prove of value in epidemiological studies on the incidence and geographic distribution of Colorado tick fever. Furthermore, if results with animal sera parallel those obtained with human sera, the tests may be applied to study the ecology of Colorado tick fever.

15747

Studies on Bacterial Resistance to Streptomycin.*

CAROLINE A. CHANDLER AND EMANUEL B. SCHOENBACH. (Introduced by P. H. Long.)

From the Department of Preventive Medicine, The Johns Hopkins University School of Medicine, Baltimore, Md.

The rapid development of resistance to streptomycin by various organisms, both *in vitro* and clinically, has recently been reported by a number of workers.¹⁻⁴ Miller and

Bohnhoff¹ have shown that gonococci and meningococci, originally susceptible to 8-40 units per cc can become resistant to concentrations of streptomycin as high as 75,000 units per cc within 4 to 6 transfers. Klein and Kimmelman^{2,3} found that with the 12 strains of *Shigella* studied, resistance was increased from concentrations of 3-7 units to

* These investigations were supported by grants from Abbott Laboratories, Eli Lilly and Company, Lederle Laboratories, Inc., Parke, Davis and Company, and the Upjohn Company.

¹ Miller, C. P., and Bohnhoff, M., *J. A. M. A.*, 1946, **130**, 485.

² Klein, M., and Kimmelman, L. J., *J. Bact.*, 1946, **51**, 581.

³ Klein, M., and Kimmelman, L. J., *J. Bact.*, 1946, **52**, 471.

⁴ Alexander, H. E., *J. Pediatrics*, 1946, **29**, 192.

1,000 units of streptomycin per cc, in from 2 to 11 transfers, depending on the particular strain. Clinically, Alexander¹ reported the isolation, from 2 patients, of 2 strains of *H. influenzae* which had the capacity to thrive in the presence of streptomycin in a concentration of 1,000 units per cc. The study reported in the present paper was undertaken to determine: (1) the ease with which resistance to streptomycin could be developed by various organisms; (2) the degree and duration of such resistance; and (3) the ability of resistant strains to grow in freshly defibrinated blood from a normal adult as compared with that of their parent, susceptible strains.

Materials and Methods. The strain of staphylococcus used throughout the tests was a *Staphylococcus aureus* (Merck test strain SM) obtained from Sydenham Hospital.[†] The strain of *H. influenzae*, type b, was isolated from a patient with influenzal meningitis in Sydenham Hospital. The pneumococcus, strain SVI, was a type I organism originally isolated in 1938 and kept since then on artificial media, with frequent passage through mice to maintain its virulence, until the beginning of the experimental period. The beta hemolytic streptococcus used was a Group A, type 14 strain obtained from Dr. Rebecca Lancefield in 1938. It was received in lyophilized form and was maintained in that state until the beginning of this study. Following initial experiments all cultures were transferred at 3- to 4-week intervals on suitable culture media not containing streptomycin. No attempt was made to maintain the original virulence of the strains during the experimental period.

The susceptibility of the strains to streptomycin was determined by the use of a series of tubes of nutrient broth containing falling concentrations of streptomycin, ranging from 125 to 6.25 μ g per cc expressed as pure base.[‡] Each tube contained 10% less

streptomycin as measured against the initial tube in a regular progression so that the interval 125 to 6.25 represented 15 tubes. Each tube was inoculated with 0.05 cc of an 18-hour broth culture in such dilution as to yield an inoculum of 200,000 to 2 million organisms per tube. The tubes were incubated at 37°C and readings were made at the end of 24 hours. End points by this method were so apparent that plating of the mixtures of organisms and streptomycin after 24 hours incubation was not necessary. When, toward the end of this study, it became necessary to enumerate bacterial colonies, streptomycin dilutions in one cc amounts were added directly to tubes containing 9 cc melted nutrient agar and after thorough mixing were poured into sterile petri dishes. Quantitated inocula of strains to be examined were then streaked on the surfaces of these plates. The number of micrograms of streptomycin in a series of plates varied from 10 to 1000 per cubic centimeter.

After the initial susceptibility of the various test strains to the action of streptomycin had been determined, each strain was transferred daily to broth tubes containing increasing concentrations of streptomycin. To provide adequate growth factors, 20% rabbit serum was added to the broth in which streptococci and pneumococci were grown, 5% Fildes' peptic digest was added to the broth in which the influenza bacilli were grown, but infusion broth alone proved adequate for the growth of the staphylococci. Control cultures containing no streptomycin were also transferred at daily intervals on all test strains.

The bactericidal power of freshly defibrinated human blood on all strains, both before and after the acquisition of resistance, was determined in parallel each time with the same blood specimen. Five hundredths cc of each 10-fold serial dilution (10^{-1} to 10^{-6}) of an 18-hour broth culture was added to 0.25 cc of freshly defibrinated human blood in each of 6 sterile pyrex tubes. These tubes were sealed, placed in a rotating box and maintained at 37°C for 24 hours. After preliminary observation, incubation without rotation was continued for another 24 hours,

[†] These strains from Sydenham Hospital were obtained through the courtesy of Dr. Horace L. Hodes and Miss Helen Zepp.

[‡] The preparation of streptomycin employed had a potency of 1000 units per milligram of pure base. (Pfizer, lot No. 4613.)

after which the tubes were opened and the contents cultured on blood agar plates. In order to determine the number of organisms added to each tube of blood, count plates were made of the 10^{-5} and 10^{-6} dilutions.

Results. (1) when tested initially, the staphylococcus was inhibited by 8 μ g of streptomycin per cc; the *H. influenzae* by 1 μ g; the pneumococcus by 37 μ g; and the streptococcus by 25 μ g per cc. After 6 daily transfers in the presence of streptomycin, the pneumococcus and the streptococcus grew in maximal concentrations of 125 μ g per cc. The staphylococcus, originally inhibited by 8 μ g, was able to multiply in 1,000 μ g per cc after 12 daily transfers. The *H. influenzae*, initially inhibited by 1 μ g, grew in 60 μ g per cc after 14 daily transfers. Unfortunately, at this time both the parent and resistant strains of *H. influenzae* were lost and further studies could not be carried out.

(2) The susceptibility to streptomycin was retested on all strains (parent, parallel control and resistant) after they had been transferred repeatedly in the absence of streptomycin for 6 months. It was found that the parent and control strains of the streptococcus, pneumococcus and staphylococcus were still inhibited by the same concentrations of the antibiotic. The resistant streptococcus, although not in contact with streptomycin for this period, had not only maintained its ability to grow in 125 μ g per cc, but actually showed an increase in resistance in that it could now grow in 500 μ g per cc. Growth was inhibited at 1,000 μ g per cc. The resistant staphylococcus had also maintained its capacity to grow in 1,000 μ g per cc and when tested in even higher concentrations, it was found to grow abundantly in 5,000 and 10,000 μ g per cc. Moderate growth was observed in the presence of even 50,000 μ g per cc. This strain was not tested at higher concentrations of the drug because the supply of streptomycin was still limited. Unlike the streptococcus and the staphylococcus, the resistant pneumococcus did not maintain its ability to grow in 125 μ g of streptomycin per cc. When retested, it was found to be inhibited by concentrations great-

er than 87 μ g per cc.

At the time of reexamination of these strains for their susceptibility or resistance to streptomycin one additional set of experiments was initiated. If resistant strains are mutants thrown off by susceptible strains, it should theoretically be possible to select them much more readily in very young cultures. Accordingly, the susceptible staphylococcus was grown in broth at 37°C for 1 hour, 2 hours and 4 hours, after which a known inoculum of each culture was put into broth to which streptomycin had been added in the following final concentrations: 0, 10, 25, 75, 100, 250, 500, 750, 1000 μ g per cc. The tubes were incubated at 37°C for 24 hours and the results were then read. All tests were run in duplicate.

It was found that with an inoculum of 200 million organisms per tube the one-hour culture was able to grow in a concentration up to 25 μ g of streptomycin. The 4-hour culture, in an inoculum of 11 million organisms per tube (the culture having been diluted to 10^{-2} , because growth was so heavy at 4 hours) grew out in 10 μ g of streptomycin per cc but not at higher concentrations. Growth was observed with the 2-hour culture with an inoculum of 400 million bacteria per tube at 10, 25, 50, 100, 500, 750 and 1000 μ g of streptomycin per cc. No growth was apparent in those tubes containing 75 μ g per cc. Thus, at a very early stage of growth it was possible, by chance selection, to isolate highly resistant mutants. When these resistant mutants were subcultured to broth containing streptomycin it was found further that they bred true in that they grew in the same concentrations or higher. Therefore with a single transfer, resistance to streptomycin in a concentration as high as 1,000 μ g per cc was obtained.

An attempt was made to explain this last observation. A series of agar plates containing 0, 10, 25, 50, 75, 100, 250, 500, 750 and 1,000 μ g of streptomycin were inoculated with 0.1 cc amounts of a 10^{-5} dilution of each of the following: (1) the original staphylococcus which grew initially in not more than 8 μ g of streptomycin; (2) the one-

TABLE I.
Deactivation of Organisms in Various Staphylococcus Cultures Growing at Indicated Streptomycin Concentrations.

Test inoculum of staphylococci	Concentration of streptomycin in medium (μg per cc)																			
	0		10		25		50		75		100		250		500		750		1000	
	No.	%	Colonies No.	%	Colonies No.	%	Colonies No.	%	Colonies No.	%	Colonies No.	%	Colonies No.	%	Colonies No.	%	Colonies No.	%	Colonies No.	%
Control culture	720	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Culture with growth at 25 μg streptomycin per cc	640	100	—	—	200	31.3	2	0.3	2	0.3	4	0.6	2	0.3	0	0	0	0	0	0
Same at 100 μg	1200	100	—	—	—	—	—	—	—	—	800	66.5	920	76.6	800	66.6	760	63.2	720	60
Same at 500 μg	700	100	—	—	—	—	—	—	—	—	—	—	—	—	700	100	600	85.7	600	85.7

hour culture which grew in not more than 25 μg ; (3) the 2-hour culture which grew at 100 μg , and (4) the 2-hour culture which was capable of growth in the presence of 500 μg . After 36 hours incubation at 37°C, colonies were observed with the original staphylococcus on the plate containing no streptomycin, but none were present on those plates containing 10 to 1,000 μg . The one-hour culture which grew initially in 25 μg showed abundant growth on plates containing streptomycin up to this level. On each of the plates containing 50, 75, 100 and 250 μg , 2 to 4 colonies grew out, indicating that approximately 1% of the inoculated organisms were resistant to higher concentrations of streptomycin while 99% were inhibited by more than 25 μg per cc. The 2-hour culture, resistant to 100 μg in broth, grew well on all plates. Colony counts indicated that most of the organisms able to grow in 100 μg could also grow in all 4 higher concentrations. Likewise, with the 2-hour culture originally resistant to 500 μg , almost all the organisms were found to grow in concentrations of 500, 750 and 1,000 μg . These results are summarized in Table I.

(3) A comparison of the ability of parent and resistant strains to grow in fresh human blood as in a bactericidal test was the third objective of this study. During the initial period of these experiments, a comparison of the ability of parent, parallel control and resistant strains to grow in normal human blood revealed no difference with the 3 organisms tested—the staphylococcus, the pneumococcus and the streptococcus. As measured by this method, the virulence or potential invasiveness of all 3 resistant strains was unchanged with respect to the virulence of the homologous parent strains.

When retested 6 months later, it was found that the virulence of the parent, control and resistant strains of the pneumococcus had diminished markedly and to the same extent. All 3 strains, parent, control and resistant, of staphylococci had, on the other hand, maintained their original virulence. Finally, in the case of the streptococcus, whereas the parent and control strains showed marked

TABLE II.
Summary of Bactericidal Studies on Streptomycin Susceptible and Resistant Strains.

Organism	Strain examined	Streptomycin resistance, μg per cc	Min. No. of organisms required to initiate growth per cc of blood	
			Initial period	Six-month re-test
<i>Pneumococcus</i>	*Parent	37	160	100,000
	Resistant	125	340	100,000
<i>Staphylococcus</i>	*Parent	8	320	500
	Resistant	1000-50,000	200	150
<i>Streptococcus</i>	*Parent	25	32	120,000
	Resistant	125-500	36	160

* Since the parallel control strains gave essentially identical results as the parent strains in every instance, they were not recorded in this table.

diminutions in virulence, the resistant strain had retained its original virulence. This observation was repeated and no evidence of a decrease in titre was found. These results are summarized in Table II.

Discussion. Streptomycin resistance can readily be induced *in vitro*. In these studies, the relative ease was found to vary with the particular organism used. With the streptococcus, pneumococcus, and *H. influenzae*, the initial resistance could be enhanced from 4 to 20 times by serial passage in streptomycin containing media. The strain of staphylococcus, originally completely inhibited by 8 μg was observed to acquire the ability to grow in the presence of more than 50,000 μg of streptomycin.

Although repeated serial passage appeared necessary to select out the resistant variants, further study with the staphylococcus revealed that in the early logarithmic stage of growth it was possible, by chance selection, to isolate highly resistant mutants in a single transfer.

The proportion of resistant organisms present in an inoculum could be estimated by seeding streptomycin containing plates. The original strain was completely inhibited by 10 μg . A strain found to be resistant to 25 μg per cc contained 33% resistant colonies at this drug level as compared with the control plate. This same strain contained 0.2% to 0.4% colonies resistant to 50, 75, 100 and 250 μg of streptomycin per cc. The proportion of colonies resistant to higher concentrations up to 1,000 μg of streptomycin was exceedingly high (60 to 100%) when the

inoculum was derived from strains found resistant to 100 μg or greater. This distribution of resistant colonies was present when serial transfer or random selection from young cultures was used as the screening procedure. In the usual method for determining streptomycin resistance, the small number of resistant organisms present is probably insufficient to initiate growth and, therefore, it may not contribute to the observed end point. The rate at which resistance is apparently acquired is a function of the proportion of resistant organisms present at each successive level. This phenomenon may also explain erratic results sometimes noted. The growth which appears at a high level of streptomycin despite the absence of growth at lower levels, may be attributed to the random selection of resistant variants.

Repeated transfer by subculture on non-streptomycin containing media over a period of 6 months did not result in a loss of streptomycin resistance, with the possible exception of one strain, the pneumococcus.

The bactericidal studies were performed as an index of virulence. No loss in potential invasiveness accompanied the acquisition of streptomycin resistance. After serial transfer of parent, parallel control and resistant strains for a period of 6 months, the ability of the pneumococci to survive in whole blood was greatly diminished while that of the staphylococci was unchanged. With the susceptible parent and parallel control strains of streptococci a marked diminution was noted, but the resistant streptococcus retained its potential invasiveness.

The general experience has been that strains which become resistant to penicillin lose their invasive properties as indicated by growth in whole blood.^{5,6} Strains which have developed resistance to sulfonamides, however, retain this capacity unchanged.⁷ From the present observations it appears that, like the latter, organisms resistant to streptomycin may also retain their virulence. In addition, such strains may preserve the ability to maintain their invasiveness upon prolonged artificial cultivation to a greater extent than the parent, nonresistant culture. The clinical implications of these observations that invasiveness may be maintained and retained are evident. The development of streptomycin resistance should be reduced to a minimum through the use of a dosage

schedule which will ensure early, adequate blood levels. In the light of these studies, an adequate level must be defined in terms of the specific susceptibility of the organism plus its potential ability to acquire resistance. The level of tolerance to streptomycin as well as the rate at which this tolerance is acquired are important factors and a large margin of safety is necessary.

Summary. 1. Resistance to streptomycin can be induced by repeated transfer of various organisms in streptomycin containing media. 2. In the early logarithmic stage of growth, highly resistant mutants can be isolated, by chance selection, in a single transfer. 3. In most instances, acquired resistance to streptomycin is maintained. 4. Organisms resistant to streptomycin may retain their original virulence as measured by the bactericidal test. 5. The acquisition of resistance to streptomycin with the maintenance of virulence may have certain therapeutic implications.

⁵ Spink, W. W., Ferris, V., and Virino, J. J., *Proc. Soc. Exp. Biol. and Med.*, 1944, **55**, 210.

⁶ Rake, G., McKee, C. M., Hambre, D. M., and Houek, C. L., *J. Immunol.*, 1944, **48**, 271.

⁷ Chandler, C. A., and Janeway, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1939, **40**, 179.

15748

Effect of Atabrine on Auricular Fibrillation in the Dog.

M. M. GERTLER AND DOROTHY KARP. (Introduced by B. P. Babkin.)

From the Department of Physiology, McGill University, Montreal, Canada.

At present there are no available data concerning the action of atabrine on cardiac muscle. However, the analogous physiological effects of quinine and atabrine in diminishing the contractibility and increasing the refractory period of striated and smooth musculature,¹ lead to a reasonable assumption that atabrine will produce similar actions to those of quinine on cardiac muscle.

Quinine and especially its more powerful isomer, quinidine, have been used for many years as a routine practice in the treatment of auricular fibrillation presumably because of their ability to prolong the refractory

period in cardiac muscle and its nodal tissues as well as to decrease myocardial excitability.² Since recent evidence has been advanced^{1,3} which clearly indicates that atabrine possesses more potent physiological and pharmacological properties when compared with quinine, it seemed most interesting to see whether atabrine would also control auricular fibrillation. Experiments in the dog were, therefore, devised to test the validity of this concept.

Methods. Five dogs were anesthetized

¹ Lewis, T., Drury, A. N., Biesen, C. C., and Wedd, A. M., *Heart*, 1921-22, **9**, 207.

² Gertler, M. M., and Karp, D., *Revue Can. de Biol.*, in press.

³ Keogh, P., and Shaw, F. H., *Australian J. Exp. Biol. and Med. Sci.*, 1944, **22**, 139.

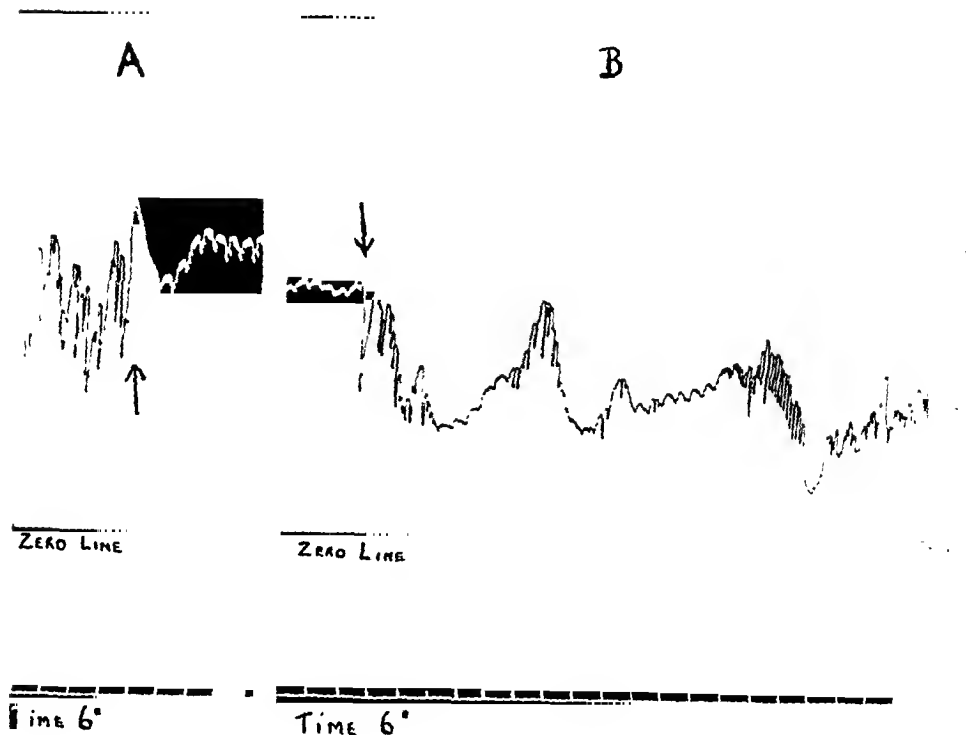


FIG. 1.

A. Restoration of normal sinus rhythm to auricular fibrillation 3 min. 24 sec. after intravenous injection of atabrine was started (Exp. 1).

B. Induction of auricular fibrillation approximately 2 hours after injection of atabrine.

with nembutal (25 mg per kg). Auricular fibrillation was produced according to the method of Hoff and Nahum.⁴ Application of mecholyl (0.2% solution in normal saline) over the sino-auricular node, followed by stimulation of any region on the right auricle with a weak faradic current consistently provoked auricular fibrillation. Auricular fibrillation produced in this manner persists for at least 10 to 15 minutes and often longer. Attempts to reproduce auricular fibrillation were made immediately after its restoration to normal sinus rhythm by atabrine. One animal received 1.0 mg of eserine (physostigmine sulphate) intravenously before such an attempt was made.

A Sanborn cardiette was employed for

electrocardiographic recordings. Lead II was used throughout the experiments with the animals placed in the dorsal recumbent position.

Atabrine dihydrochloride (Winthrop Chemical Co.), a 0.5% solution in normal saline, was always freshly prepared for these experiments.

Electrocardiographic and blood pressure records were taken immediately prior to and during auricular fibrillation. These records were continued during the infusion of atabrine and continued thereafter until the heart returned to its normal rhythm.

Experimental Results. The restoration of regular sinus rhythm to auricular fibrillation occurred rapidly in all the 5 animals following the intravenous infusion of atabrine (Table I). The gradual recovery to normal rhythm in Experiment 1 can be seen in the

⁴ Hoff, H. E., and Nahum, L. H., *Am. J. Physiol.*, 1940, 129, 428.

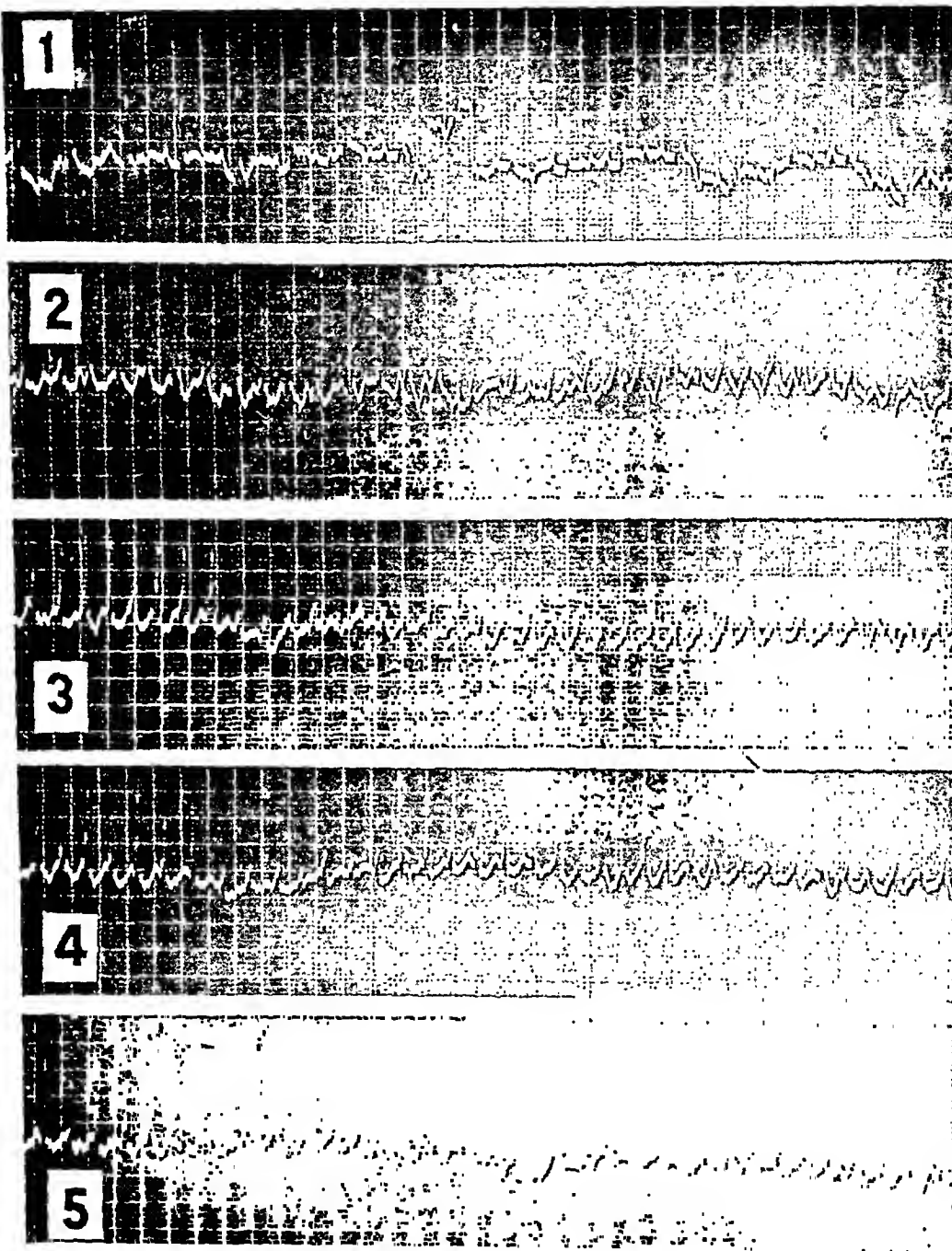


FIG. 2.

Electrocardiogram. Lead II. Same experiment as in Fig. 1. 1—Control record. Heart rate 150 at 10:30 a.m. 2—Auricular fibrillation. Heart rate 234 to 312 at 10:35 a.m. 3—Infusion of atabrine started. 4—5 mg atabrine injected. 5—5 mg atabrine injected.

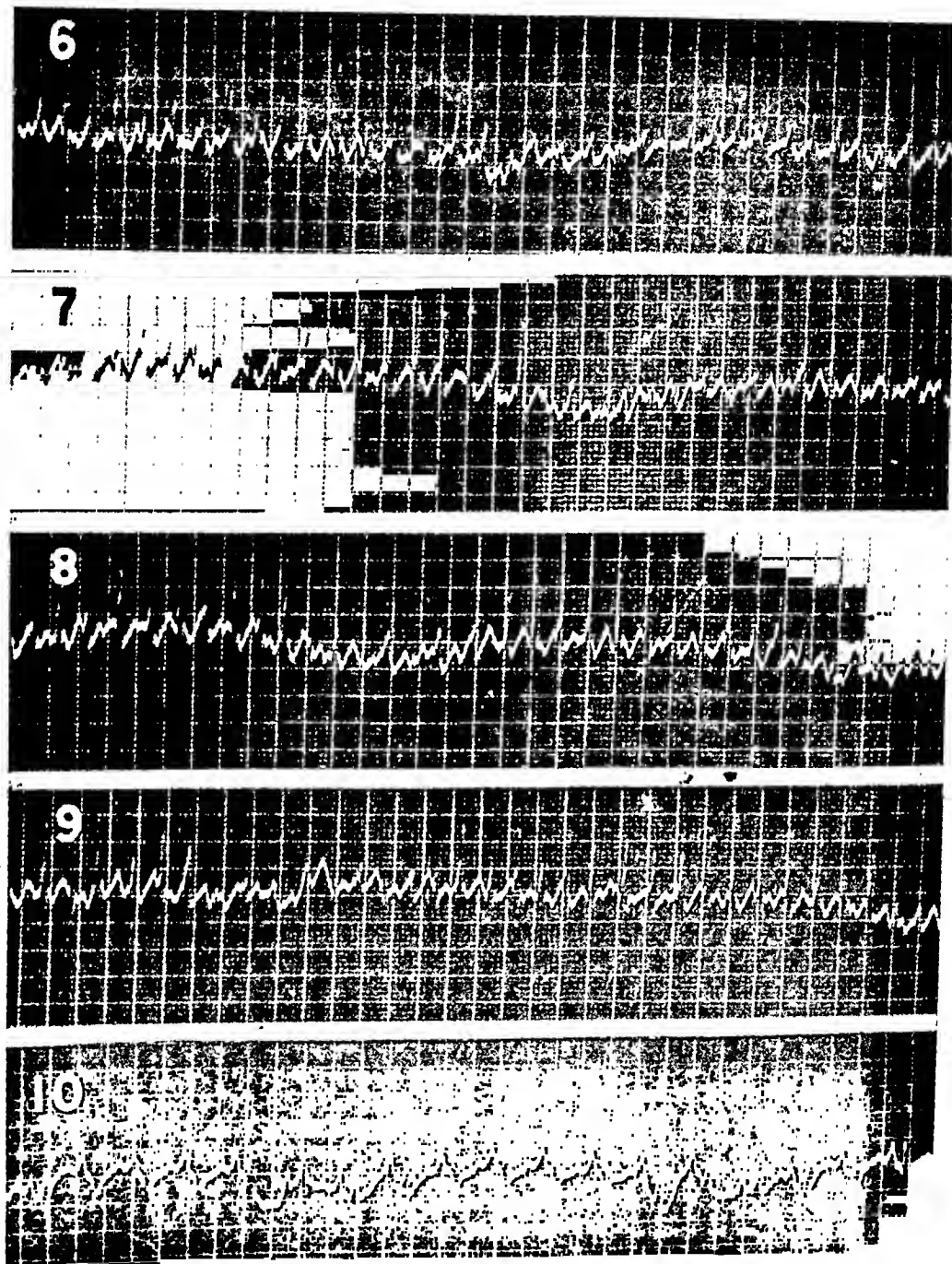


FIG. 3.

Electrocardiogram, Lead II. Continuation of Fig. 2. 6—5 mg atabrine injected. 7—5 mg atabrine injected. 8—5 mg atabrine injected. 9—7.5 mg atabrine injected. 10—Return to normal rhythm after total injection of 37.5 mg. Heart rate 167 at 10:40 a.m. See Fig. 1A.

TABLE I.

Exp. No.	Wt of dog (kg)	Total amt of atabrine (mg)	Mg/kg
1	15	30.0	2.0
2	26	37.5	1.44
3	13.6	40.0	2.94
4	12.5	33.5	2.65
5	8	22.5	2.81

kymographic and electrocardiographic records (Fig. 1, 2 and 3).

Several phenomena were observed in the ventricles. During the early period of atabrine injection the ventricles contracted with great irregularity which disappeared as the concentration of atabrine in the circulating blood was increased by virtue of the continuous infusion. As the infusion of atabrine was continued, the usual accompanying variations in the amplitude of the R wave became less pronounced despite the persistent auricular fibrillation (Fig. 2 and 3). It was also observed that intracardiac injection of atabrine failed to restore the fibrillating ventricle in 2 of the experiments.

The effect of atabrine was long-lasting. Attempts to reproduce auricular fibrillation after its arrest by atabrine were unsuccessful for approximately 2 hours in 2 experiments. In the remaining 3 experiments where auricular fibrillation was reproduced successfully, it was necessary to combine the application of mecholyl pledgets in the region of the S-A node with faradic stimulation of the right auricle for at least 30 seconds. The auricular fibrillation produced by this method was transient, rarely exceeding more than 20 seconds.

Discussion. Experimental evidence for another possible clinical application of atabrine has been brought out in the study of atabrine on the dog heart. The need for a drug in the treatment of auricular fibrillation which is as effective as digitalis or quinidine and which is not as toxic is obvious. It is noteworthy that atabrine, a drug which is efficacious in malaria therapy and which has proven itself to be virtually harmless by its long clinical record, should also control auricular fibrillation. After a survey of the literature, the only reference to atabrine and auricular fibrillation found was that made by

Ganguli.⁵ While studying the effects of atabrine on the electrocardiogram, in patients with malaria, he included one case which suffered from auricular fibrillation concomitant with malaria. The final electrocardiographic record did not reveal auricular fibrillation, but whether this may be attributed to atabrine or to the conventional forms of therapy cannot be stated with certainty from his article.

Obviously, any drug which reconverts auricular fibrillation into normal sinus rhythm must of necessity increase the refractory period of the "circus movements" in the auricular muscle and diminish the rate of conduction in these circuits.⁴ No attempt was made in these experiments to analyze the mechanism by which atabrine restored regular sinus rhythm to auricular fibrillation. The auricular fibrillation produced in these experiments was of the vagal type, *viz.* increased vagal tone plus superimposed auricular excitation. It would be reasonable, therefore, to explain the action of atabrine in restoring regular sinus rhythm to this type of auricular fibrillation on the basis that atabrine paralyzes the vagal inhibitory fibers to the dog heart.³ However, this explanation is not entirely adequate, for it is known that atabrine possesses such properties as decreasing the excitability of excised auricular tissue,⁶ perfused hearts⁷ and increasing the refractory period in skeletal muscle.¹

In order to test the validity of whether the beneficial effect of atabrine in auricular fibrillation was merely due to its *antivagal* action, it was decided to inject eserine into an animal immediately after atabrine had restored regular sinus rhythm to the experimentally produced auricular fibrillation and observe whether it was possible to reproduce the fibrillation. It was found that auricular fibrillation could be produced with great difficulty and persisted for only 10 seconds. Furthermore, an exclusive *antivagal* effect is

⁵ Ganguli, P., *Arch. f. Schiffs-u-Trop. Hyg.*, 1933, 37, 413.

⁶ Suffolk and Berkshire, *Earl of. Quart. J. Exp. Physiol.*, 1939, 29, 1.

⁷ Chin, K., *Japan J. Med. Sci., IV, Pharm.*, 1937, 10, 162P.

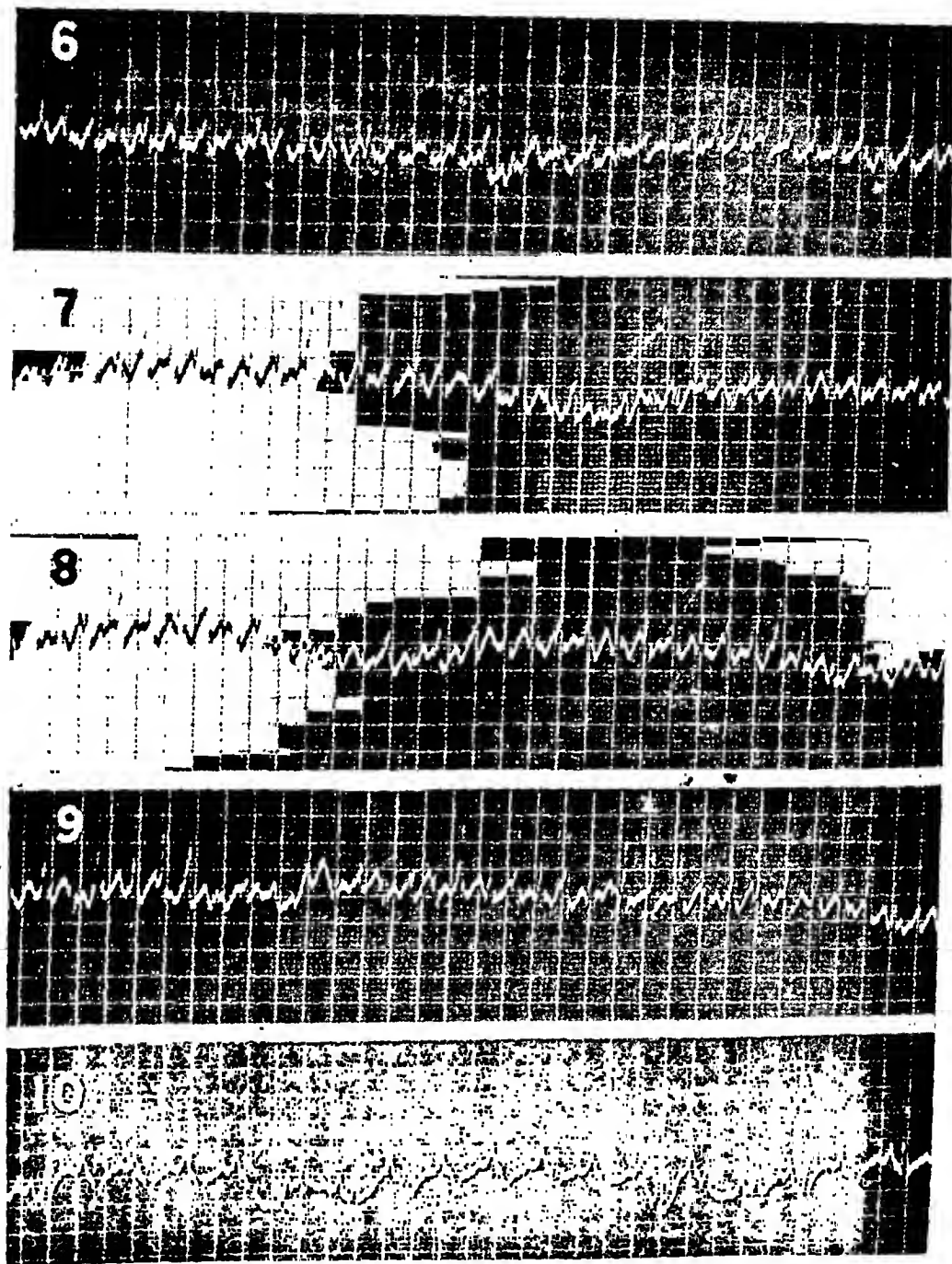


FIG. 3.

Electrocardiogram. Lead II. Continuation of Fig. 2. 6—5 mg atabrine injected. 7—5 mg atabrine injected. 8—5 mg atabrine injected. 9—7.5 mg atabrine injected. 10—Return to normal rhythm after total injection of 37.5 mg. Heart rate 167 at 10:40 a.m. See Fig. 1A.

TABLE I.
Effect of Penicillin on the Production of Hemolysis, Lethal Factor and Dermonecrotin by Staphylococci.

Toxic factor	Staphylococcus strains	Medium					
		Antigen liquid		Antigen agar		Veal infusion agar	
		Penicillin	No penicillin	Penicillin	No penicillin	Penicillin	No penicillin
Rabbit cell hemolysin (MHU/ml)†	161 N		64		128		
	161 R		32-64	64	64	8	64
	147 N	<2	32-64	128	128	128	128
	196 N	<2	32-64	128	128	64	256
	43 N		32		64		128
	43 R	4	16	16	8	32	64
Sheep cell hemolysin (MHU/ml)†	161 N		<2		0		
	161 R	0	0	8	0	0	8
	147 N	0		<2	<2	0	0
	196 N	<2	32	32	128	64	512
	43 N		0		8		
	43 R	0	0	0	0		
Lethal factor	161 N		4/4 (1)		4/4 (.5)		4/4 (.5)
	161 R	0/4 (1.0)‡	4/4 (1)	0/4 (1.0)	4/4 (.5)	4/4 (.5)	4/4 (.5)
	147 N	0/4 (1.0)	4/4 (1)	4/4 (0.5)	4/4 (.5)	4/4 (.5)	4/4 (.5)
	196 N	0/4 (1.0)	4/4 (1)	4/4 (0.5)	4/4 (.5)	4/4 (.5)	4/4 (.5)
	43 N		4/4 (1)		4/4 (.5)		4/4 (.5)
	43 R	0/4 (1.0)	3/4 (1)	4/4 (1.0)	4/4 (1.0)	4/4 (.5)	4/4 (.5)
Dermonecrotin	161 N		+				
	161 R	0	+				
	147 N	±	+				
	196 N	0	+				
	43 N		+				
	43 R	0	±				

* N = normal; R = artificially resistant strains. Strains, except No. 43, are enterotoxigenic.

† MHU (Minimal Hemolytic Dose) of hemolysin indicates least amount of toxin which lyses completely 1.0 ml of a 1% suspension of erythrocytes after incubation for 1 hour at 37°C (rabbit r.b.c.) followed by overnight refrigeration (sheep r.b.c.).

‡ Numerator—number of mice killed within 48 hours; denominator—number mice injected; parentheses figures—dosage in ml.

§ + = area of necrosis and redness 5 mm or more in diameter; ± = area of redness <5 mm; 0 = no reaction.

opposed by the fact that it was impossible to reproduce auricular fibrillation in 2 experiments for a long period after its reconversion into normal sinus rhythm by atabrine despite the presence of a normal electrocardiogram.

While there is no experimental proof as yet, it is reasonable to assume from the evidence submitted that the mechanism of the action of atabrine on auricular fibrillation is similar to that of quinine and quinidine.

Summary. 1. Experimentally produced auricular fibrillation in the dog was success-

fully restored to normal sinus rhythm by the intravenous infusion of atabrine (average 2.17 mg per kg). 2. The mechanism by which atabrine might produce this effect is discussed. It is suggested that its action is similar to that of quinine or quinidine.

The authors are indebted to Dr. B. P. Babkin for suggesting this problem and for his advice and unflinching interest. To Dr. H. E. Hoff the authors are grateful for his valuable advice and criticism.

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Effect of Penicillin on Growth and Toxin Production of Enterotoxigenic Staphylococci.*

MILTON SEGALOVE AND K. EILEEN HITE. (Introduced by G. M. Dack.)

From the Department of Bacteriology and Parasitology, the University of Chicago.

Little is known of the effect of penicillin on formation or activity of bacterial toxins. Ercoli, Lewis and Moench¹ demonstrated *in vitro* neutralization of diphtheria toxin-antitoxin mixtures: Boor and Miller^{2,3} obtained protection *in vivo* against meningococcal and gonococcal endotoxin and Mason⁴ demonstrated inhibition of plasma coagulation by enterotoxigenic staphylococci. Neter⁵ failed to obtain neutralization of tetanus toxin and Blair, Carr and Buchman⁶ found no inhibition of plasma coagulation and formation of hemolysin by staphylococci.

The present paper reports experiments on

* This work was supported in part by a grant from the National Canners Association.

¹ Ercoli, N., Lewis, M. N., and Moench, L. J., *J. Pharm. and Exp. Therap.*, 1945, **84**, 120.

² Boor, A. K., and Miller, C. P., *Science*, 1945, **102**, 427.

³ Miller, C. P., and Boor, A. K., *Proc. Soc. E.P. Biol. and Med.*, 1946, **61**, 18.

⁴ Mason, H. C., *J. Immunol., Virus Res. and Exp. Chemother.*, 1945, **51**, 307.

⁵ Neter, E., *J. Infect. Dis.*, 1945, **76**, 20.

⁶ Blair, J. E., Carr, M., and Buchman, J., *J. Immunol., Virus Res. and Exp. Chemother.*, 1945, **52**, 281.

the effect of penicillin on formation of staphylococcal enterotoxin, hemolysins, lethal factor and dermonecrotxin.

Tested by the tube dilution method,⁷ the natural resistance of 15 enterotoxigenic strains of staphylococci was found to range from 0.05 units/ml to 500 units/ml of penicillin. Three enterotoxigenic (No. 147, 161, 196) and one nonenterotoxigenic (No. 43) strains used in the following experiments were naturally resistant to 500, 30, 100 and 0.1 units/ml of penicillin respectively. The resistance of strains 161 and 43 was further increased to 2000 and 10 units/ml respectively by growth in increasing penicillin concentrations. All strains were alpha hemolytic and strain 196 produced potent beta lysin.

Toxin production in the presence and absence of penicillin was tested in semisolid (0.7%) veal infusion agar and in liquid and semisolid media containing 1% Amigen,[†] 0.25% glucose, 1.2 µg/ml nicotinic acid, 0.05

⁷ Kolmer, J. A., *Penicillin Therapy*, D. Appleton-Century Co., Inc., New York, N.Y., 1945

[†] Pancreatic hydrolysate of casein free of vitamins and carbohydrate. Product of Mead, Johnson and Co.

thermal requirements, responses, and availability.

Although it is stated in the literature¹ that reptiles do not respond to tetanus toxin, experience in the field and laboratory suggested that there was great possibility of error in the previous experiment due to lack of appreciation of the vital necessity of maintaining optimum thermal condition. In most experimental work with reptiles, the full importance of the temperature factor has not been recognized.

In the pilot experiment, because reptiles had been reported as not susceptible to tetanus toxin, the arbitrary enormous dose of 20,000 guinea pig M.L.D.'s (minimal lethal dose) was employed in testing a lizard weighing 65 g. It was planned that, in case the symptoms of tetanus did not appear within a few days' time, the animals were to be exposed to optimum temperatures of 34°C to 40°C in the hope that during the approach towards or into this normal temperature range, the onset of symptoms might reveal a critical threshold at which neural and muscular effects might develop and below which partial cold narcosis might prevent their appearance.

Contrary to expectation, the original and all subsequent experiments demonstrated the fact that these animals, when held at temperatures ranging from 10°C through to the optimum of 38°C, are by no means immune to the effects of tetanus toxin although at all low temperatures there is a marked retardation in the appearance of symptoms.

Exp. I. Three specimens of *Dipsosaurus dorsalis dorsalis* were given the following doses: 20,000 guinea pig M.L.D.'s in one instance, for the other 2, 10,000 guinea pig M.L.D.'s each. The toxin was injected intramuscularly in the femoral regions in these and all subsequent experiments. The animals were then maintained at temperatures of 24°C to 26°C until the onset of symptoms. In all instances first symptoms appeared as slight tremors in the head and tail regions. These tremors appeared first in the individual receiving 20,000 guinea pig M.L.D.'s. The

first perceptible tremors occurred 36½ hours after inoculation. Two hours after onset of primary symptoms, action of the hind legs was impaired and the tail was arched strongly in a lateral position.

This specimen which had received 20,000 guinea pig M.L.D.'s was heated to 32°C at which time opisthotonic contractions arched the tail and back to a dorsolateral position. Sound or sudden movements taking place in close proximity to the animal caused convulsions, although fairly periodic spasms were observed even without extra stimulation. Three hours after the appearance of symptoms this individual was placed in an incubator refrigerator at a temperature of 10°C-11°C and was maintained under these conditions until death occurred 212 hours later.

The remaining 2 subjects which had been given 10,000 guinea pig M.L.D.'s, only half the dosage administered to the first lizard, showed these initial tremors 40 hours after injection. One of these was immediately placed in a temperature of 10°C-11°C while the other was maintained at room temperature. At room temperature (27°C-28°C) death took place between 72 and 80 hours after inoculation. Prior to death, violent symptoms of tetanic convulsions and opisthotonic contraction threw the animal into a strong sigmoid curve with the head inclined upward at a sharp angle. This posture is retained with only slight relaxation even after death. (Fig. 2).

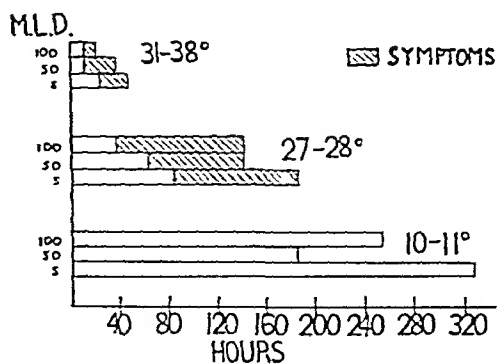


Fig. 1.

Duration of life in desert iguana, injected with tetanus toxin, with respect to maintenance temperature.

¹ Metchnikoff, E., *Ann. Inst. Past.*, 1897, **11**, 501.

$\mu\text{g/ml}$ thiamine HCl, 1.0 $\mu\text{g/ml}$ calcium pantothenate and inorganic salts.⁵ All media were adjusted to pH 7.6. Cultures were incubated at 37°C for 3 days. Strains 161 R, 147 N, 196 N and 43 R were grown in 1000, 200, 50 and 5 units/ml of penicillin respectively. Toxins were produced and assays of enterotoxin and hemolysin were carried out by previously described methods.⁹⁻¹¹ Lethal factor was detected by the intravenous injection of mice; dermonecrotxin by intracutaneous injection of rabbits with 0.1 ml of a 1:20 dilution of toxin in saline.

Counts of viable bacteria revealed no difference in the amount of growth in media containing penicillin compared to the same media without penicillin.

Positive tests for enterotoxin^{9,10} were obtained in monkeys or cats with centrifugates

⁸ Surgalla, M. J., and Hite, K. E., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61**, 244.

⁹ Havisson, E., Duck, G. M., and Cary, W. E., *J. Infect. Dis.*, 1938, **62**, 219.

¹⁰ Duck, G. M., *Food Poisoning*, University of Chicago Press, 1943.

¹¹ Surgalla, M. J., and Hite, K. E., *J. Infect. Dis.*, 1945, **76**, 78.

of enterotoxic cultures grown in all of the above media with and without penicillin.

Results of assays for hemolysins, lethal factor and dermonecrotxin are summarized in Table I. The potency of the hemolysins, lethal factor and dermonecrotxin produced by strains naturally or artificially resistant to penicillin was markedly reduced in cultures grown in Amigen liquid containing penicillin. However, in semisolid Amigen and veal infusion agar cultures, questionable effect of penicillin was noted. Penicillin in the same concentration failed to neutralize preformed hemolysins, lethal factor and dermonecrotxin.

Summary. The natural resistance of 15 enterotoxic strains of staphylococci to penicillin was found to range from 0.05 to 500 units per ml. Production of staphylococcal hemolysins, lethal factor and dermonecrotxin, but not of enterotoxin, was inhibited by growing selected naturally and artificially resistant strains in Amigen liquid medium containing sublethal concentrations of penicillin. Inhibition was not observed in similar experiments using semisolid Amigen and veal infusion media.

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Studies on Thermal Sedation in Suppression of the Symptoms of Tetanus Toxin

R. B. COWLES AND NORMAN B. NELSON. (Introduced by A. J. Salle.)

From the Departments of Zoology, Public Health and Preventive Medicine, University of California, Los Angeles.

The following experiments on the physiological effects of temperature suggest that cold narcosis, as seen in reptiles and some warm-blooded vertebrates, will modify the development of symptoms resulting from tetanus toxin. It is possible that cold narcosis might be used as a method of sedation and as an adjunct to therapy in such diseases as rabies and tetanus.

Because of the wide range in temperature tolerance exhibited by the diurnal reptiles

and because of their similarity to the mammals with respect to optimum temperature, which in many lizards range from 36°C to 41°C, and because of their thermal simplicity and the greater facility with which these animals can be utilized in temperature experiments, it was agreed that any common diurnal species of reptiles would be suitable for experimentation, and the northern crested lizard *Dipsosaurus dorsalis dorsalis* was selected as being most convenient as to size,

relation of violence of reaction with temperature change is in harmony with the anticipated results and is also in accord with the generally known effect of temperature changes with respect to metabolic rates in general.

Exp. II. The extreme sensitivity of these reptiles to tetanus toxin suggested that a second series of lizards be tested with lighter dosages in order that some approximation to their M.L.D.'s might be obtained prior to any additional expenditure of living material. For this reason, similar treatment was utilized but dosages of 100, 800 and 4,000 guinea pig M.L.D.'s were administered to the animals.

Following inoculation, the lizards were first maintained at 26-28°C; that is, 10° below their optimum. Thus they were held under conditions which seemingly would retard slightly the onset of symptoms.

Although all 3 lizards appeared normal for the first 48 hours, that with 4,000 units showed extreme symptoms 75 hours after inoculation. The 800 guinea pig M.L.D. individual displayed only the primary evidence of involvement, tremors of the head and cervical regions. All 3 animals were placed in the refrigerator incubator at 10°C as soon as symptoms were detected. The 100-unit individual showed no symptoms and was continued at room temperatures pending development of evidence of toxic effect.

After 90 hours at 26-28°C, the individual with 100 guinea pig M.L.D.'s was found to be suffering from partial paralysis of the hind legs and was then placed with the other 2 individuals at a temperature of 10°C.

Death of the 4,000-unit individual occurred sometime between 196 to 214 hours after inoculation, the time lapse probably being somewhat curtailed owing to a 2-hour period of warming to 31°C while photographs were being taken to illustrate symptoms.

The remaining 2 individuals, those at 800 and 100 units, while still refrigerated, showed marked opisthotonic contractions up to 244 hours, these symptoms gradually diminishing in intensity until immobility, possibly from prolonged exposure to abnormally low temperature, concealed the reaction. These con-

ditions led imperceptibly to death some 270 hours (± 12 hours) after inoculation.

Exp. III. In this investigation, an attempt was made to accomplish the following:

1. Test more fully the retarding effects of low temperature.

2. Test the accelerating effect of optimum temperature.

3. Determine the effect of small dosages of the toxin on:

- a. Animals continuously cooled to torpidity (10°C) from the time of inoculation to the natural termination of the experiment.

- b. A series maintained at temperatures of 27-28°C, and

- c. A series at temperatures slightly above the optimum but within voluntary tolerance range.

4. Determine the approximate lizard M.L.D. for this species under 3 conditions: namely,

- a. When cooled to torpidity.

- b. Maintained at approximately minimum-activity level, and

- c. At and slightly above the optimum.

Procedure. Nine lizards were divided into 3 groups, one of which received 100 M.L.D.'s, another 50 guinea pig M.L.D.'s, and the third 5 guinea pig M.L.D.'s, thus reducing the dosage from that of the maximum administered in the first experiment to 1/200, 1/400, and 1/4,000 of that massive inoculation. From each of these dosage groups, one individual was placed in the incubator-refrigerator at 10°C, one in a cage at room temperature 26-28°C, and a third was placed in a heating cabinet at 38°C, the approximate optimum temperature, although still 5°C below temperatures selected by these animals under natural conditions and some 7-9° below the lethal temperature for the species. Throughout the first 17 hours in a heating cabinet, temperatures were raised slowly from 31-38°C and thereafter were maintained at the latter level.

In order to postulate the exact relationship between different thermal levels and the progress of the intoxication, larger series of animals as well as greater standardization of

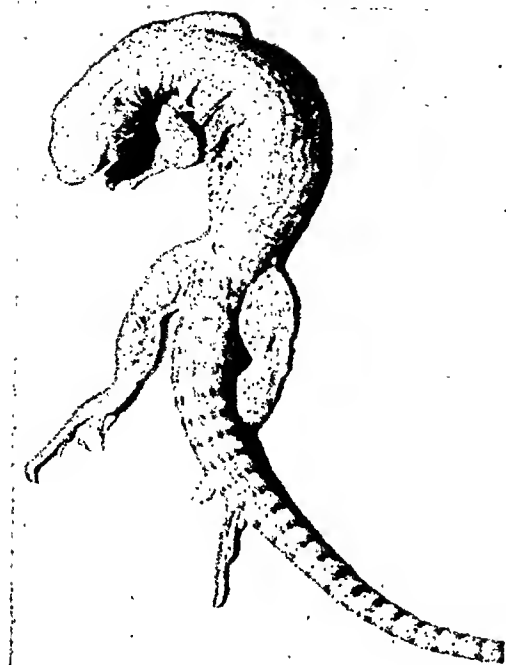


FIG. 2.

Dead *Diposaurus* showing characteristic posture attendant on death from tetanus.

Eighty hours after inoculation, the third specimen, which had been maintained at 10°C , was removed from the cold chamber and allowed to warm to room temperature, in this case 25.5 to 27°C . On warming, locomotory movements were limited to forelimbs but movement was poorly coordinated. The hind legs were immobilized and were strongly adpressed to the tail, although locomotion could be carried on with the front legs alone.

As temperatures rose from 25.5 - 27°C , convulsive, tetanic contractions became progressively more violent and, as reported above, it was noticeable that auditory and visual stimuli, as well as vibrations and movements taking place in close proximity to the animal precipitated these convulsive reactions.

At 28°C the body became still more violently flexed, remaining in this position almost continuously. The flexure was so violent that the animal was forced to lie on one side. At 30°C the head was flexed strongly to the left and the forelimbs showed limited mobility and were flexed backwards

under the body. After being exposed to warming temperatures for 23 minutes, the animal defecated for the first time and the body temperature was noted as being 31°C . Twenty-five minutes after removal from the cold chamber and while still with body temperature of 31°C , it was first noted that respiratory movement had ceased, but it is possible that respiration may have stopped shortly before.

Concomitant with cessation of respiration, the general reactions became fewer and less severe and the animal remained in a contorted, prostrate position. Only 52 minutes after being removed from the control chamber, and with body temperature having risen to only 31.5°C , the animal died.

Animal No. I, which received double the dosage given the other 2 individuals, was maintained continuously at a temperature of 10°C thus acting as a control for No. II and III. In spite of such a heavy dosage, this animal outlived the others by a total of 212 hours, whereas, the individual chilled for 80 hours then warmed to 32°C died after only 52 minutes at a moderate temperature for these animals. Since the optimum temperature for this species approximates 37°C and may rise to 43°C with no discomfort to the animal, it is clearly apparent that death in so short a time cannot be attributed to overheating or overstimulation. At least one factor causing death from tetanus toxin seems to be the violence of contraction, thus with approach toward normal neuromuscular reactions, the toxin-produced reaction may cause traumatic injury and thus death. It seems improbable that either the amount or the degree of diffusion of the neurotoxic element can have caused death where no symptoms were evoked; on the contrary, this would appear to be a case of death induced by some other toxic effect or by too prolonged chilling, which seems rather improbable, or some other unknown factor.

In these experiments the most clearly discernible effect of temperature is the very marked lowering of the intensity of all tetanic reactions and their gradual intensification attendant on rising temperatures. This cor-

acidotic dogs; (2) that the administration of thiosulfate *per se* does not significantly disturb either glomerular filtration rate or renal plasma flow; and (3) that the administration of thiosulfate does not greatly alter the rate of excretion of titratable acid. Accordingly, the thiosulfate clearance is to be preferred to the creatinine clearance in studies on renal function in animals in acidosis.

Methods. A total of 12 experiments were performed on 3 trained female dogs rendered acidotic by the oral administration of ammonium chloride. Solutions containing creatinine, sodium thiosulfate and sodium *p*-aminohippurate were infused at a constant rate into the saphenous vein. Except in Experiments 1 and 2 the rate of infusion was maintained at 3 cc per minute. In these initial experiments infusion rates of 10 cc per minute gave evidence of expansion of circulating blood volume and increased filtration rate. High infusion rates were therefore discontinued. Plasma and urine thiosulfate concentrations were determined by the plasma method of Newman *et al.*² Corrections were made for both plasma and urine blanks. Other procedures and chemical methods have been described in previous communications.^{3,4}

Identity of the creatinine and thiosulfate clearances in acidotic dogs. In 50 comparisons of simultaneously determined thiosulfate and creatinine clearances in 3 acidotic dogs, the mean thiosulfate:creatinine clearance ratio was 1.004. In 10 experiments the ratios varied within limits of 0.95 and 1.05. In 2 experiments ratios as low as 0.90 and as high as 1.13 were observed. It is evident within limits of experimental error that the thiosulfate and creatinine clearances are equal in acidotic dogs as they are in normal dogs.¹

In Table I are presented 2 illustrative experiments in which the plasma concentration of thiosulfate was varied from 17.8 to 55.6 mg %. The plasma concentration of creatinine was maintained essentially constant at an optimum level for the determination of filtration rate. It is obvious that the 2 clear-

TABLE I.
Comparisons of the Thiosulfate and Creatinine Clearances in the Acidotic Dog Over a Range of Plasma Thiosulfate Concentrations.

Exp. No.	Dog No.	Total concentration, min.	Arterial plasma concentration			Urine		Rate of excretion		Clearance		Ratio, Thio./Cre.
			pH	Bicarbonate, mM/l	Creatinine, mg%	Thio-sulfate, mg%	Flow, cc/min.	pH	Titratable acid, mEq./min.	Ammonia, mEq./min.	Thio-sulfate, cc/min.	
1	1	80-90	7.31	13.6	27.8	19.5	2.0	5.05	0.065	0.062	64.0	0.96
		90-100			26.3	18.3	2.0	5.01	0.074	0.071	74.3	0.98
		115-125	7.31	13.9	25.5	35.3	10.9	5.00	0.079	0.075	80.4	1.03
		125-135			25.3	34.7	10.2	5.09	0.070	0.071	79.5	1.01
2	2	150-160	7.33	13.9	25.6	52.0	11.9	5.34	0.053	0.069	81.3	1.02
		160-170			26.4	51.1	11.9	5.32	0.054	0.069	80.8	1.03
		110-120	7.29	11.0	27.4	18.6	5.6	5.10	0.060	0.085	61.3	0.98
		120-130			26.6	17.8	7.5	5.06	0.063	0.087	63.9	0.97
3	3	150-160	7.29	11.7	27.4	36.1	11.3	5.17	0.061	0.083	68.8	1.03
		160-170			28.0	35.9	8.6	5.17	0.060	0.081	67.0	1.03
		185-195	7.31	11.6	28.9	54.8	8.0	5.24	0.057	0.085	66.1	1.04
		195-205			29.1	55.6	7.8	5.27	0.053	0.083	65.5	1.00

⁴ Pitts, R. F., and Lotspeich, W. D., *Am. J. Physiol.*, 1946, **147**, 138.

procedures would have been required, conditions not compatible with objectives in these preliminary experiments.

In spite of the exploratory nature of these tentative trials, it is apparent that the life expectancy of these lizards, when under normal thermal conditions—that is, 31–38°C—is less than 2 days; whereas with moderate cooling to 27–28°C or chilling to 10–11°C, there is a material prolongation of life, in the former case by 7 to 8 days, and in the latter as much as 14 days. Unfortunately, controls were not run to determine how long a normal lizard would survive at this temperature (ice box).

Although these results might have been predicted by the use of Van't Hoff's Law, the ameliorating effects are greater than the Q_{10} of 2, and approach those of 3 to 4. This larger factor may be due to inherent differ-

ences of tissues or to physiological functions, but at present it seems more probable that the explanation may lie in the realm of normal differences of the Q_{10} for different thermal levels.

Conclusions. 1. Contrary to reports indicating that "cold blooded" creatures are not susceptible to tetanus toxin, the desert iguana is susceptible and for temperature studies is an excellent experimental animal. 2. Lowering of temperature markedly prolongs life in the desert iguana inoculated with tetanus toxin. From Van't Hoff's Law one would expect a slowing down of the effects of the toxin at lower temperatures. 3. There is a tremendous field awaiting study on the effects of altering bodily temperature on the course of disease, especially where toxic factors are concerned.

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Use of Thiosulfate Clearance As a Measure of Glomerular Filtration Rate in Acidotic Dogs.*

R. F. PITTS AND W. D. LOTSPEICH.

From the Department of Physiology, Syracuse University Medical College, Syracuse, N.Y.

The renal clearance of thiosulfate has recently been proposed as a measure of glomerular filtration rate in the normal dog¹ and in man.² Since the majority of the ionic constituents of the glomerular filtrate are more or less completely reabsorbed during their passage through the renal tubules, the apparent tubular rejection of this anion constitutes an interesting anomaly. The present study of the excretion of thiosulfate in the acidotic dog was undertaken with the following 2 ends in view. If thiosulfate were to undergo no reabsorption in the tubules

of animals with disturbed acid base relationships, as is apparently true in normal animals, that fact would strengthen the concept that its clearance constitutes an adequate and broadly applicable measure of filtration rate. If the thiosulfate clearance could be used as a measure of filtration rate in acidotic animals, it would have the following advantage over the creatinine clearance. Thiosulfate, unlike creatinine, is devoid of buffer properties. Hence, it can be predicted that the administration of this substance would not affect the rate of excretion of titratable acid, as does the administration of creatinine.³

In this study it has been found: (1) that the thiosulfate clearance provides an accurate means of quantitating glomerular function in

* Aided by grants from the John and Mary R. Markle Foundation and from the United States Public Health Service.

¹ Gilman, A., Philips, F. S., and Koelle, E., *Am. J. Physiol.*, 1946, **146**, 348.

² Newman, E. V., Gilman, A., and Philips, F. S., *Bull. Johns Hopkins Hosp.*, 1946, **79**, 229.

³ Pitts, R. F., and Alexander, R. S., *Am. J. Physiol.*, 1945, **144**, 239.

ances are in essential agreement, and that the thiosulfate clearance is independent of plasma level over a 3-fold range of concentration. For these reasons we feel that the thiosulfate clearance gives as accurate an estimate of the quantity of fluid filtered through the glomeruli as does the creatinine clearance. The severity of the acidosis in the animals used in these experiments is indicated both by the low arterial pH values, and by the reduced plasma bicarbonate concentrations. As a consequence of the acidosis the urines were highly acid and ammonia and titratable acid were excreted at accelerated rates. It is significant that the buffering capacity of the urinary creatinine accounts for over 90% of the titratable acid eliminated.

Lack of effect of thiosulfate on glomerular filtration rate and minimum effective renal plasma flow. It is apparent from Experiments 3 and 4 in Table II, that the administration of thiosulfate is without significant effect on filtration rate and minimum effective renal plasma flow. Throughout both experiments these renal variables were measured respectively by the creatinine clearance and by the *p*-aminohippurate clearance. The initial 3 periods of each experiment constitute the control observations. During the final 3 periods thiosulfate was infused at such a rate that its clearance could be used as an accurate measure of filtration rate. No really significant change occurred in either filtration rate or renal plasma flow as a result of the infusion of thiosulfate, slight increases in Experiment 3 being offset by slight decreases in Experiment 4. Identical experiments on 2 other animals yielded similar results.

Advantage of thiosulfate over creatinine in experiments in which titratable acid is to be measured. It was noted in connection with Experiments 1 and 2 that over 90% of the observed urinary titratable acid could be ascribed to the buffering action of creatinine. Experiments 5 and 6 emphasize the fact that the administration of creatinine very markedly increases the rate of elimination of titratable acid in the acidotic animal. In the initial 3 periods of each experiment little buffer substance was present in the urine, and

as a consequence the rate of excretion of titratable acid was low, amounting only to 0.005 to 0.008 milliequivalents per minute. Following the administration of creatinine the rate of excretion of titratable acid increased more than 10 times to values ranging from 0.085 to 0.105 milliequivalents per minute. Since the urinary pH did not change, the increased elimination of titratable acid derived solely from the increased elimination of creatinine.

Experiments 3 and 4 in Table II illustrate an interesting phenomenon which we have occasionally observed, namely, that the urine may not be especially acid even though the extent of the reduction of the alkali reserve is great (see especially Experiment 4). The excretion of titratable acid was moderate in this experiment although the urine contained much buffer. Following the administration of thiosulfate, the urine pH fell and the excretion of titratable acid increased proportionally. This effect on urine pH is not peculiar to thiosulfate for we have observed it following the administration of sodium phosphate, sodium *p*-aminohippurate and even sodium chloride in the dog. At the moment we have no adequate explanation for it. It is by no means universally observed.

Summary and Conclusions. The data presented above confirm the observations of Gilman *et al.*¹ that the thiosulfate clearance may be used as a valid measure of glomerular filtration rate in the dog, and extend these observations by showing that it is applicable to animals with disturbed acid base relationships as well as to normal animals. Because the administration of thiosulfate has no significant effect on glomerular filtration rate (creatinine clearance) and minimum effective renal plasma flow (*p*-aminohippurate clearance) the thiosulfate clearance may be used in studies on renal function, in acidotic animals with minimal disturbance of these discrete renal variables. Since thiosulfate is not a buffer, the administration of this substance does not greatly alter the rate of excretion of titratable acid. This virtue is likewise possessed by mannitol and inulin, but the simplicity and accuracy of the thiosulfate analysis argues strongly in its favor.

TABLE II.
Experiments Illustrating the Lack of Effect of the Administration of Thiosulfate on Glomerular Filtration Rate and Minimum Effective Renal Plasma Flow in the Acidotic Dog.

Exp. No.	Dog No.	Total concurrent time, min.	Arterial plasma conc.										Clearance										Clearance ratio	
			pH	Bicar- bonate, mM/l	Crea- tinine, mg%	Thio- sulfate, mg%	Urine		Rate of excretion		Clearance		Crea- tinine, mg/min.	Thio- sulfate, mg/min.	p-Amino hip- purate, mg/min.	Crea- tinine, mg/min.	Thio- sulfate, mg/min.	p-Amino hip- purate, mg/min.	Cr.	P.A.H.	P.A.H.			
							Flow, cc/min.	pH	mEq./min.	acid, mEq./min.	Ammonia, mEq./min.	Crea- tinine, mg/min.										Thio- sulfate, mg/min.	p-Amino hip- purate, mg/min.	
3	1	110-120 120-130 130-140	7.20	9.8	29.9 29.4 30.2	51.5 51.3 52.4	1.05 1.12 1.19	2.6 2.4 2.1	5.31 5.26 5.19	.048 .050 .050	.077 .075 .081	74.4 71.1 74.1	248 234 238					.30 .30 .31						
		170-180 180-190 190-200	7.22	10.0	30.6 30.5 30.6	30.6 32.4 32.4	1.38 1.29 1.22	2.0 2.5 2.4	4.99 4.98 4.99	.090 .098 .088	.079 .079 .076	75.4 78.9 78.9	238 265 268					.32 .30 .30						
4	1	115-125 125-135 135-145	7.33	14.5	23.7 23.8 24.0	28.2 25.9 24.9	1.69 1.69 1.69	2.9 5.4 6.5	5.72 6.10 6.26	.021 .010 .008	.069 .055 .048	74.2 71.6 74.8	234 217 221					.32 .33 .34						
		165-175 175-185 185-195	7.36	15.1	24.5 25.1 25.1	28.2 25.9 24.9	1.75 1.73 1.68	2.9 1.5 1.2	5.00 4.92 4.86	.064 .070 .078	.074 .076 .075	68.3 68.1 73.2	197 198 217					.35 .34 .34						
5	1	100-110 110-120 120-130	7.27	11.4		41.9 42.4 41.7	1.63 1.72 1.70	3.8 2.0 1.3	5.37 5.25 4.84	.006 .005 .007	.092 .093 .094	77.1 71.3 74.0	228 208 219					.34 .34 .34						
		150-160 160-170 170-180	7.27	11.6	36.3 35.9 36.5	39.7 38.9 38.7	1.59 1.58 1.58	8.0 10.9 5.2	5.08 4.95 5.08	.087 .106 .088	.085 .084 .080	76.5 78.4 81.0	232 233 232					.33 .34 .35						
6	1	90-100 100-110 110-120	7.29	14.6		41.7 39.8 38.7	1.46 1.46 1.47	7.5 3.6 1.6	4.71 4.84 4.69	.008 .076 .008	.076 .074 .074	71.8 70.4 65.9	219 215 195					.33 .33 .34						
		120-130 130-140 140-150	7.29	14.4	27.9 28.3 28.9	37.8 37.6 38.4	1.62 1.61 1.62	1.9 4.3 4.7	4.92 4.88 4.87	.085 .088 .094	.078 .078 .076	73.5 70.7 73.9	213 214 213					.35 .33 .35						

a



b

Fig. 1a.

Return extrasystoles which appeared during warming of the auriculo-ventricular node.

Fig. 1b.

Return extrasystoles during warming of the auriculo-ventricular node, appearing regularly with a long R-P interval.

TABLE I.

Experiment of Feb. 28, Fig. 1b		Experiment of Feb. 28, Fig. 1a		Experiment of April 18	
R-P	P-R	R-P	P-R	R-P	P-R
0	0	12	19	0	0
0	0	14	17	13	10
9	0	11	20	12	0
16	10	11	21	14	9
0	0	11	21	8	0
0	0	10	22	9	15
0	0	9	24	0	0
0	0	10	22	16	9
0	0	9	24	0	0
24	9	7	0	0	0
12	0	7	0	7	0
11	0	7	0	4	0
10	0	8	0	6	0
10	0	8	0	0	0
11	0	10	24		
10	0	7	0		
12	16	7	0		
12	17				
10	0	13	0		
0	0	14	20		
0	0	12	0		
		4	0		
		14	20		
		12	25		
		14	22		
		0	0		
		0	0		

the R-P interval reached a length of 0.12 second. Premature contractions did not follow any other beat. Therefore a reentry mechanism can be assumed.

The tracings from the 2 other experiments of this series showed the same picture.

Table I gives the measurements obtained in 3 tracings with return extrasystoles. They show clearly that a return extrasystole oc-

curs when the R-P distance reaches a certain length. The reciprocal duration of the R-P distance and of the following R-P interval is evident. All beats in which the P waves were hidden in the QRS complex or in which positive P waves preceded the QRS complex are designated with "O". Following beats of this type return extrasystoles were absent, for obvious reasons.

Return Extrasystoles.*

DAVID SCHERF.

From the Department of Medicine, New York Medical College, New York City.

In previous reports it has been demonstrated that "return extrasystoles" are observed in dogs during auriculo-ventricular rhythm and simultaneous vagus stimulation or following fatigue of the conduction system by a short lasting tachycardia.^{1,2} The resulting disturbance of rhythm closely resembled tracings which were registered from patients in rare cases.^{3,4} Whenever the auricles, during auriculo-ventricular rhythm, contracted at a certain time *after* the QRS complexes the return extrasystole was observed.

Of importance is the answer to the problem whether the longitudinal dissociation is always present as an intrinsic quality of the specific conduction system between auricle and ventricle so that the reentry mechanism works whenever the R-P interval reaches a certain length or whether some damage of the conduction system must be added. This would consist in the experiments just mentioned, in vagal stimulation or fatigue. An answer may be possible when more experimental or clinical instances of this arrhythmia become known, particularly when more conditions are studied under which this disturbance of rhythm occurs.

In view of the paucity of experimental observations on this subject 2 more situations, in which the phenomenon of return extrasystoles was observed will be described in this report. It was seen (1) during direct warming of the auriculo-ventricular node of the dog's heart *in situ* by a thermode, and (2) during depression of the sinus and the

auriculo-ventricular node by mechanical stimulation of the sinus node area.

(1) *Warming of the auriculo-ventricular node.* These experiments were described in a previous communication in this journal.⁵

The wall of the right auricle was opened after clamping both venae cavae; the area of the auriculo-ventricular node near the orifice of the coronary sinus vein was warmed with a thermode. The warming took place within 30 seconds after clamping of the veins was started, that is, at a time when a high degree of anoxia could not have as yet developed.

This experiment was performed in 17 dogs. Return extrasystoles were observed each time in these experiments when an auriculo-ventricular rhythm appeared in which the auricles contracted after the ventricles during the warming. This happened 4 times.

Fig. 1a shows a tracing obtained in such an experiment. Before the warming the vagi were divided in the neck. During the warming a relatively slow auriculo-ventricular rhythm appeared. Deep, peaked inverted P waves are visible after the QRS complexes. Whenever the auricles contracted 0.14 second or later after the ventricles, a return extrasystole, due to a reentry mechanism appeared. The intraventricular conduction of the return extrasystoles was aberrant. Such extrasystoles appear after the second, fifth, sixth and seventh auriculo-ventricular beat in the tracing. Such return extrasystoles were absent after all the other beats unless the R-P interval was long.

Fig. 1b shows the same phenomenon in another experiment of the same type. The warming caused at first a tachycardia and the P waves were not visible. Here again return extrasystoles appeared when the auricles contracted after the ventricles and

* A portion of the costs was defrayed by a grant from the United Hospital Fund.

1 Scherf, D., and Shookhoff, C., *Wien Arch. inn. Med.*, 1926, **12**, 501.

2 Scherf, D., *Arch. int. Med.*, 1941, **67**, 372.

3 Drury, A. N., *Heart*, 1924, **11**, 405.

4 Decherd, G., and Ruskin, A., *Texas Rep. Biol. and Med.*, 1943, **1**, 299.

5 Scherf, D., *Proc. Soc. Exp. Biol. and Med.*, 1944, **56**, 220.

Stimulation of Sporogenic and Nonsporogenic Bacteria by Traces of Penicillin or Streptomycin

HAROLD R. CURRAN AND FRED R. EVANS. (Introduced by G. E. Holm.)

From the Division of Dairy Research Laboratories, Bureau of Dairy Industry, Agricultural Research Administration, U. S. Department of Agriculture.

The remarkable therapeutic efficacy of penicillin and streptomycin is dependent upon their low toxicity to tissues and their unusual capacity to inhibit or kill, *in vivo*, a wide variety of pathogenic organisms.

That the biological activity of these antibacterial agents is not limited to their bactericidal or bacteriostatic properties may be readily demonstrated, although this fact seems to have been overlooked by most microbiologists and clinicians. The bacteria-controlling property of these drugs has for the most part obscured the phenomenon of growth stimulation associated with low concentrations of the drugs. Evidence of a so-called biphasic action of penicillin has been reported by Miller, Green, and Kitchen¹ who noted that certain concentrations of penicillin in broth containing *Staphylococcus aureus* induced upon incubation, increased turbidity over the control containing no penicillin. The effect was irregular at 37°C but quite consistent at 24°C.

It is the purpose of this paper to show that suitably diluted solutions of either penicillin or streptomycin, when incorporated in a nutrient medium, exert a definite stimulating effect upon the growth of many bacterial species. Stimulation of this nature was first observed in a spore-forming species with tomatin as the test substance; our observations were then extended to penicillin and streptomycin and widened to include a rather large group of spore-forming species and 2 nonspore-forming pathogenic species.

When spore-forming species were used, washed spores were seeded uniformly into tubes of sterile (autoclaved) skim milk to yield a final concentration of approximately 50,000 viable spores per milliliter. The in-

oculated samples were heated at 95°C for 15 minutes and cooled, after which distilled water dilutions of the antibiotics*† were thoroughly mixed with the inoculated samples. These samples together with the controls, which received no antibiotics but were manipulated similarly, were then incubated, usually at 30°C and observed at frequent intervals for the first visible indication of growth. In the nonsporing species a small loopful of a 17-hour tomato broth culture served as inoculum for each tube of medium. The requisite dilutions of reagents were made up from the powder just prior to use. Two or more controls were included in each series to take account of chance variations. The data were essentially reproducible although in some samples unexplained discrepancies occurred.

Examination of the data (Table I) shows that both penicillin and streptomycin were for most strains stimulatory at one or more concentration levels. The degree of stimulation varied from a few hours to several days as measured by first visible evidence of growth‡ between the test and control samples. Film preparations made at one or more stages in the development of the cultures, in most instances revealed a positive correlation between visible indication of stimulation and cell numbers. Greatest stimulation was found in *Bacillus megatherium*, while in *Bacillus mycoides* and certain strains of *Bacillus cereus* it was not apparent. In *Clostridium perfringens* (2 cultures) stimulation was slight and frequently irregular. The

* Penicillin sodium obtained from Chas. Pfizer & Co., Brooklyn, N.Y.

† Streptomycin hydrochloride obtained from Merek & Co., Rahway, N.J.

‡ Perceptible thickening, surface ring of peptonized milk or gas bubble.

¹ Miller, W. S., Green, C. A., and Kitchen, H., *Nature*, 1945, 155, 210.

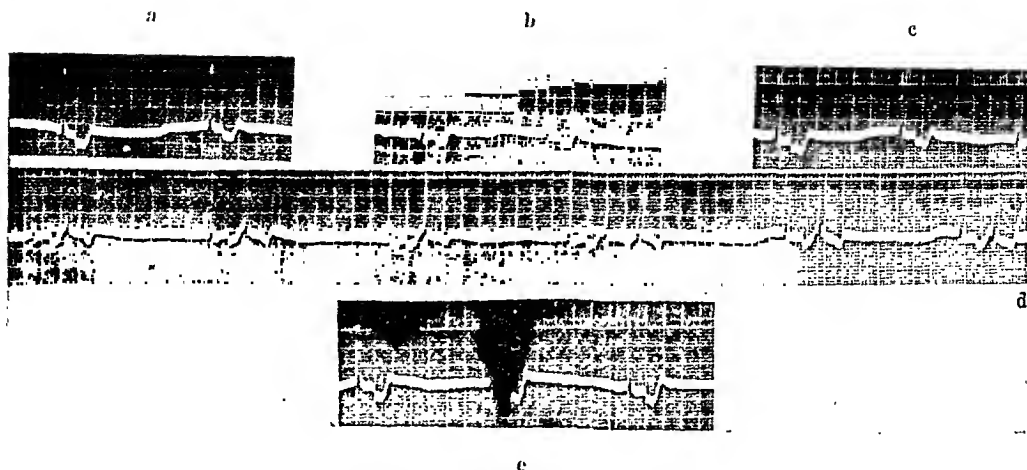


FIG. 2.

Return extrasystoles during reflex inhibition of the sinus- and the auriculo-ventricular node.

(2) *Depression of sinus and auriculo-ventricular node.* This phenomenon was described in a previous paper in this journal.⁶ The subepicardial injection of hypertonic solutions of sodium chloride or calcium chloride, as well as digitalis or strophanthin in the sinus node area of the right auricle led in a certain percentage of experiments on dogs to a depression of the sinus node. Frequently the deeper auriculo-ventricular nodal centers were also inhibited. This depression was particularly pronounced in one experiment in which during the auriculo-ventricular rhythm the auricles contracted *after* the ventricles.

The heart of the dog was exposed during nembutal-morphine anesthesia. Both vagi were severed in the neck. Then 0.1 cc of a 5% solution of calcium chloride was injected subepicardially in the area of the head of the sinus node. Immediately a marked bradycardia appeared and an auriculo-ventricular rhythm was registered with an R-P interval of 0.16 second (Fig. 2a). Without any other means the length of the R-P distance gradually increased in the following minutes. In Fig. 2b it is 0.17 second and in Fig. 2c it measures 0.18 second. As soon as it reached the value of 0.20 second,

bigeminy and trigeminy appeared (Fig. 2d). About 2 minutes later and 3.5 minutes after the injection, the extrasystoles disappeared and the R-P interval at that time was again shortened to 0.18 second (Fig. 2e). The occurrence of trigeminy due to return extrasystoles was described before.²

Discussion. These experiments show 2 new ways which lead to the appearance of return extrasystoles. In both, however, a change of the condition of the auriculo-ventricular conduction system cannot be completely excluded. This damage could be due to anoxia in the first experiments in which the exposed auriculo-ventricular node was warmed although it is rather improbable in view of the early appearance of the return extrasystoles and the shortness of the P-R and R-P intervals. In the second type of experiment the depression of the nodal centers may also have extended to the deeper sections of the specific tissue.

Conclusion. Two new methods are described which lead to the appearance of return extrasystoles during auriculo-ventricular rhythm in the dog. Such extrasystoles appeared during direct warming of the exposed auriculo-ventricular node *in situ* and during depression of the nodal centers by the subepicardial injection of a hypertonic solution of calcium chloride.

⁶ Seherf, D., Proc. Soc. Exp. Biol. and Med., 1946, 61, 286.

with those here reported indicate that the stimulating activity of penicillin and streptomycin evident *in vitro* is probably operative *in vivo*.

Discussion. By evincing, in suitable concentrations, bacteriostatic or bactericidal activity and at certain much lower concentration levels a stimulatory effect, penicillin and streptomycin conform to a familiar behavior pattern of drugs and chemical reagents. The biphasic action of sulphonamides has been reported.¹ Recognition of the potential stimulatory activity of these drugs further increases the problems of initial dosage and maintenance in the body fluids of proper drug levels. The importance of achieving complete destruction of the infective agent by the initial dosages receives added emphasis. It is apparent also that concentrations of these drugs efficacious as bactericidal or bacteriostatic agents for one species of micro-organism, for another, perhaps coexistent infective agent, may be stimulatory. Thus, Eagle *et al.*² found that 0.01-0.02 u./ml of penicillin was actively spirochaeticidal, a zone not infrequently stimulating to the spore-forming species employed in our study.

² Eagle, H., Magnuson, H. J., and Fleischman, R., *Johns Hop. Hosp. Bull.*, 1946, **79**, 168.

Rammelkamp and Keefer⁴ reported maximum antibacterial action for *Streptococcus haemolyticus* when the concentration of penicillin in blood and serum was 0.019 u./ml. Evidence^{5,6} seems to indicate that part of the therapeutic activity of penicillin is associated with accompanying impurities, a fact which has necessitated in recent years the administration of larger doses to offset the greater refinement of the product; conversely it is not unlikely that the stimulatory concentration level of penicillin and streptomycin would be lowered by increased purification.

Summary. Suitable, low concentrations of penicillin or streptomycin in sterile milk (autoclaved) stimulated the growth of many spore-formers and *Staphylococcus aureus* and *Streptococcus agalactiae*. The degree of stimulation differed with the organism and with some cultures was not apparent in the range of concentrations used; some of the practical implications of growth stimulation by penicillin and streptomycin are briefly discussed.

⁴ Rammelkamp, C. H., and Keefer, C. S., *J. Clin. Invest.*, 1943, **22**, 649.

⁵ Smith, W. J., *Science*, 1946, **104**, 411.

⁶ Comm. Med. Res., U. S. P. H., and F. D. A., *J. Med. Assn.*, 1946, **131**, 271.

15754

Studies on Auricular Tachycardia Caused by Aconitine Administration.

DAVID SCHERF.

From the Department of Medicine, New York Medical College.

Aconitine in minute amounts provokes abnormal stimulus formation in the heart. It has been demonstrated that with particular precautions a long-lasting, regular, bigeminal rhythm can be obtained after the intravenous administration of aconitine in the experimental animal.¹ Studies on the effect of

topical application of aconitine on the dog's heart are reported in this paper.

Method. The experiments were performed on 23 dogs. Morphine-nembutal anesthesia was used. The heart was exposed in the usual way. The vagi were severed in the neck. If necessary, the peripheral end was stimulated with the aid of a Cambridge inductorium.

The electrocardiograms were registered in lead II.

* Aided by a grant from the United Hospitals Fund.

¹ Scherf, D., *Z. f. d. ges. exp. Med.*, 1929, **65**, 198, 222.

TABLE I.
Effect of Traces of Penicillin or Streptomycin upon the Growth of Bacteria in Milk.

Organism	No.	Incubation temp., °C	Penicillin u/ml				Streptomycin u/ml			
			0.01	0.001	0.0001	0.00001	0.05	0.005	0.0005	
<i>B. cereus</i>	369	30	—	+ 2 hr	+ 2 hr	+	+	+	+	8 hr
"	401	30	0	+ 3 hr	+ 3 hr	+	0	+	+	0
"	232	30	+ sl	+ 3 hr	0	0	0	+	+	0
<i>B. mycoides</i>	6462	30	0	0	0	0	—	0	0	—
<i>B. megatherium</i>	696	30	+	+ 3 day ⁴	+	0	+	+	+	+
<i>B. subtilis</i>	L.B.	30	+	+ 4 hr	+	0	+	+	+	+
"	6051	30	—	—	—	—	+	+	+	3 days
" <i>atrymus</i>	230	30	—	—	—	—	+	+	+	6 hr
<i>B. stearothermophilus</i>	1518	52	—	—	—	—	+	+	+	4 hr
"	67	52	—	—	—	—	+	+	+	+
<i>Cl. perfringens</i>	846	37	—	—	—	—	+	+	+	+
"	3626	37	—	0	0	+	+	+	+	4 hr
"	3679*	37	+ sl	+ 1-2 days	+	0	+	+	+	sl
<i>Strep. agalactiae</i>		37	—	—	—	—	+	+	+	+
<i>Staph. aureus</i>		37	—	—	—	—	+	+	+	+

* A proteolytic anaerobe.

0 = first visible evidence of growth approx. equal in time to, — = greater than (retardation), and + = less than control (stimulation). For the level of concentration which showed maximum stimulation, the time has been indicated.

2 nonspore-formers,[§] *Streptococcus agalactiae* and *Staphylococcus aureus*, were chosen because of their causal relationship to bovine mastitis. The latter evidenced rather slight and at times irregular stimulation at the 0.001 and 0.0001 levels (u/ml) of penicillin and in most of the streptomycin concentrations. *Strep. agalactiae* was appreciably stimulated through a wider range of penicillin and streptomycin. In general, streptomycin was more frequently stimulatory than was penicillin. It is perhaps significant that the stimulatory effects of penicillin and streptomycin were generally more apparent in the cultures incubated at 30°C than at higher temperatures, which accords with the previously mentioned observation of Miller *et al.*¹ upon the staphylococcus.

In view of the limited number of drug levels used, it should not be supposed that the most favorable concentrations were attained in these experiments or that cultures negative in reaction would not respond at other drug levels. The recorded data were obtained in a skim milk medium; when this medium was enriched by the addition of glucose and yeast extract the stimulating properties of the antibiotics were usually greatly reduced or entirely eliminated.

Welch, Price, and Randall² recently reported that streptomycin at certain concentration levels when injected intraperitoneally in mice infected with *Eberthella typhosa* increased rather than decreased the fatality rate. This suggested to the authors the possibility of stimulation of the infective agent by streptomycin; further strengthening this view was their observation that certain levels of streptomycin in broth, though inhibiting any visible evidence of growth, induced, nevertheless, a greater multiplication of cells than did samples containing lower concentrations of the drug. This report, together with that of Miller *et al.*,¹ came to our attention after most of our observations were completed. The former observations together

§ Recently isolated from acute bovine mastitis by L. A. Burkey, this Bureau.

² Welch, H., Price, C. W., and Randall, W. A., *J. Am. Pharm. Assn.*, 1946, **35**, 155.

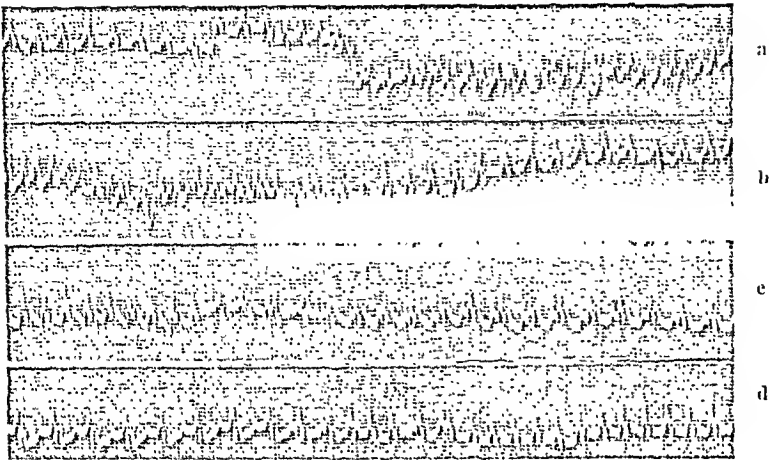
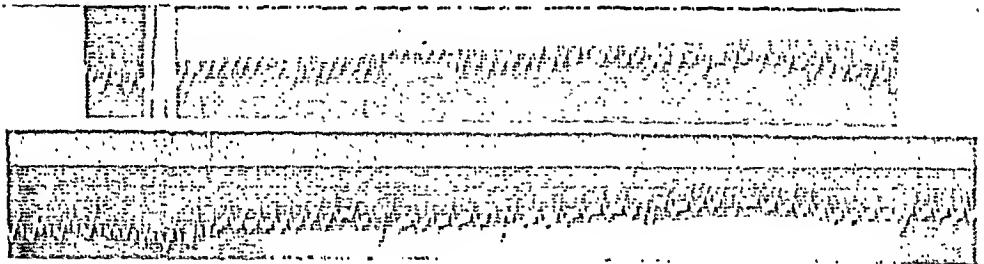


FIG. 1, a-d.

The top tracing (Fig. 1a) shows at the beginning an auricular tachycardia and the effect of vagus stimulation which begins in the middle of the tracing. Fig. 1b shows the end of the vagus stimulation (middle of tracing). In the beginning of tracing 1c the site of stimulus formation was clamped off; in the middle of Fig. 1d the clamp was removed (Lead II).

a



b

FIG. 2a and 2b.

The signal indicates the beginning of vagus stimulation. Fig. 2b shows a slight irregularity of the auricle during vagus stimulation (Lead II).

tinuation of Fig. 1a). Without delay the same tachycardia with a rate of 230 was again registered as in the beginning of Fig. 1a. In the first third of Fig. 1c, the appendix of the left auricle was clamped, so that the site of injection was isolated from the rest of the heart. A sinus rhythm appeared immediately with a rate of 187 beats per minute and soon the rate fell to 156. The clamp was removed a few seconds later (middle of Fig. 1d, which is a direct continuation of Fig. 1c), and an auricular tachycardia reappeared with a rate of 250 beats per minute.

Fig. 2a shows at the beginning a few complexes of a tachycardia with a rate of 200 which appeared following an injection of the same quantity of aconitine into the medial wall of the left auricular appendix. The broad, wide, irregular line represents a signal indicating the beginning of stimulation of the left vagus nerve. During the stimulation of the vagus the auricular rate rose immediately to 461 and there was almost complete inhibition of the auriculo-ventricular conduction. The cessation of vagus stimulation was followed by an immediate recurrence of the

Solutions of 0.05% of "potent" aconitine crystals "Merck" were used. The injections were performed with greatest care with the aid of a tuberculin syringe. Even the small amounts of aconitine, which were used in these experiments, if introduced into the auricular cavity, caused a ventricular tachycardia and ventricular fibrillation. One must be careful not to leave any aconitine solution on that part of the auricular surface which is in contact with the ventricular wall because this also leads to a ventricular tachycardia and eventually to ventricular fibrillation. The potency of the solution quickly deteriorates, even when kept in the refrigerator.

Most of the injections were performed on the appendix of the left auricle, a few millimeters below its tip. Injections into the wall of the appendix of the right auricle had the same result.

Experimental results. Injection of 0.05 cc of a solution of 0.05% of aconitine into the wall of the auricles of the dog's heart leads to a prolonged regular auricular tachycardia. The tachycardia begun within 90 seconds after the injection. The rate varied between 196 and 310 per minute. The tachycardia lasted between 40 and 60 minutes, and was therefore sufficiently long to permit certain studies to be made.

In these experiments the following facts could be demonstrated:

1. Faradic stimulation of the right or left vagus nerve during the auricular tachycardia led to a remarkable acceleration of the rate of the auricle in all experiments. This acceleration often amounted to an increase of more than 100%. The acceleration was immediate, and after the end of stimulation, the former rate returned within a few seconds in the form of a rapid but not abrupt decline. The auricular waves remained unchanged in the electrocardiogram, or showed only the slight alterations which would be expected with an increase in rate. No difference could be detected in the results when right and left vagus were stimulated respectively. Observation of the right auricle during the vagus stimulation revealed an imme-

diately disappearance of the strong fluttering movements, and on close observation, weaker, but rapid and regular undulatory movements could be detected. A varying degree of auriculo-ventricular block appeared. Auricular fibrillation, or rapid reexcitation of the auricles with rates up to 3500 per minute was never observed. In only one experiment did auricular fibrillation appear following the injection of aconitine and before stimulation of the vagi.

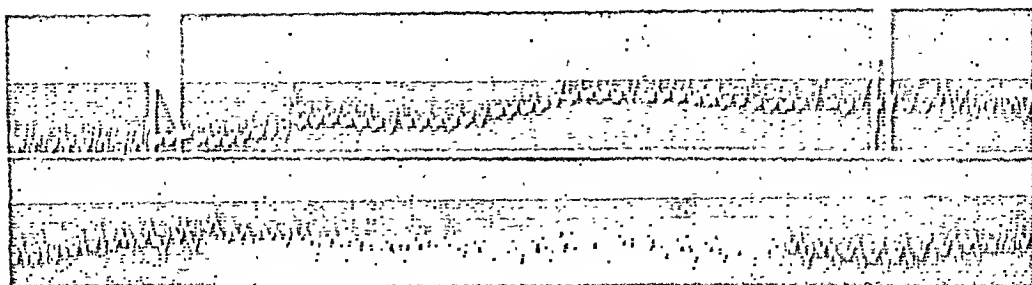
2. Clamping off the appendix of the auricle into which the injection was made, invariably led to an immediate disappearance of the tachycardia and the reappearance of sinus rhythm. In some instances coronary sinus rhythm with deep inverted P waves was noted; this was soon followed by regular sinus rhythm. Removal of the clamp permitted the tachycardia to reappear within a few seconds.

3. Stimulation of the vagus while the clamp was in position led to a complete cardiac standstill. Only in the clamped-off appendix of the left auricle were fine undulatory movements visible.

4. Cooling the focus of origin of the tachycardia, that is the area where the injection had been made, with a thermode also caused the tachycardia to vanish for the duration of the cooling. The tachycardia recurred immediately and invariably with the removal of the thermode.

Fig. 1a shows at the beginning a regular tachycardia caused by the injection of aconitine in the manner described in the appendix of the left auricle. A rate of 230 auricular and ventricular beats per minute is present. The R waves are so thin that they are scarcely visible. The P waves are tall. After the 10th beat the left vagus was stimulated in the neck with a strong faradic current. The auricular rate immediately increased to 428 beats per minute without any change in the form of the auricular waves. An auriculo-ventricular block appeared and made it easier to observe the auricular electrocardiogram. The vagal stimulation was stopped a few seconds later (first third of the tracing in Fig. 1b, which is a direct con-

a



b

FIG. 4a and 4b.
Vagus stimulation during auricular tachycardia (Fig. 4a) and cooling of area of injection (Fig. 4b) (Lead II).

cooling led to the appearance of sinus rhythm. The response to cooling was, in general, the same as to clamping off of the area of injection.

In most experiments all these measures, such as vagus stimulation, clamping, and cooling, were performed 3 or 4 times in succession, and always with the same results.

Discussion. Of paramount importance in the evaluation of these experiments is the question as to whether the tachycardias caused by the injection of aconitine represent an auricular tachycardia of the "essential paroxysmal tachycardia" type, or whether we are dealing with auricular flutter. This differentiation is often impossible and in recent years many arguments have been presented in favor of the identity of these conditions. It is the opinion of the author that for the time being a separation is necessary, and that the facts in favor of their identity are too few and not really crucial.

The tracings appear similar to those published by many authors as examples of experimental auricular flutter.²⁻⁵ The isoelec-

tric line between the single auricular waves is short or absent. Flutter rates in the dog's auricles however, were found to vary between 345 and 580⁶ while the rate in the experiments reported in this paper was definitely below this range before vagus stimulation and within it during stimulation. Against the diagnosis of a simple tachycardia like the essential paroxysmal tachycardia in man there is the observation that stimulation of either vagus nerve with even the strongest faradic current never caused sudden ending of the tachycardia. This sudden abolition of an auricular tachycardia is seen however in only a certain percentage of essential paroxysmal tachycardia, and absence of this effect does not invalidate the diagnosis. It is entirely possible that the inability to end the tachycardia by vagal stimulation is due to the special conditions which led to the tachycardia in this experiment. Against the existence of auricular flutter in these tracings, the argument may be used that it was never possible, even with the strongest current, to change the auricular tachycardia into auricular fibrillation by vagus stimulation. One easily succeeds in doing this in auricular flutter caused by other means.^{4,7} The phenomenon of rapid reexcitation with auricular rates between 1500 and 3500 per minute rarely occurs during vagus stimulation

² Lewis, T., Drury, A. N., and Bulger, H. A., *Heart*, 1921, 8, 83.

³ Lewis, T., Drury, A. N., and Bulger, H. A., *Heart*, 1921, 8, 141.

⁴ Rothberger, C. J., and Winterberg, H., *Arch. f. d. ges. Physiol.*, 1914, 160, 42.

⁵ Scherf, D., *Z. f. d. ges. exp. Med.*, 1928, 61, 30.

⁶ Lewis, T., Feil, H. S., and Stroud, W. J., *Heart*, 1918:20, 7, 191.

⁷ Lewis, T., Drury, A. N., and Biesseu, C. C., *Heart*, 1921, 8, 311.

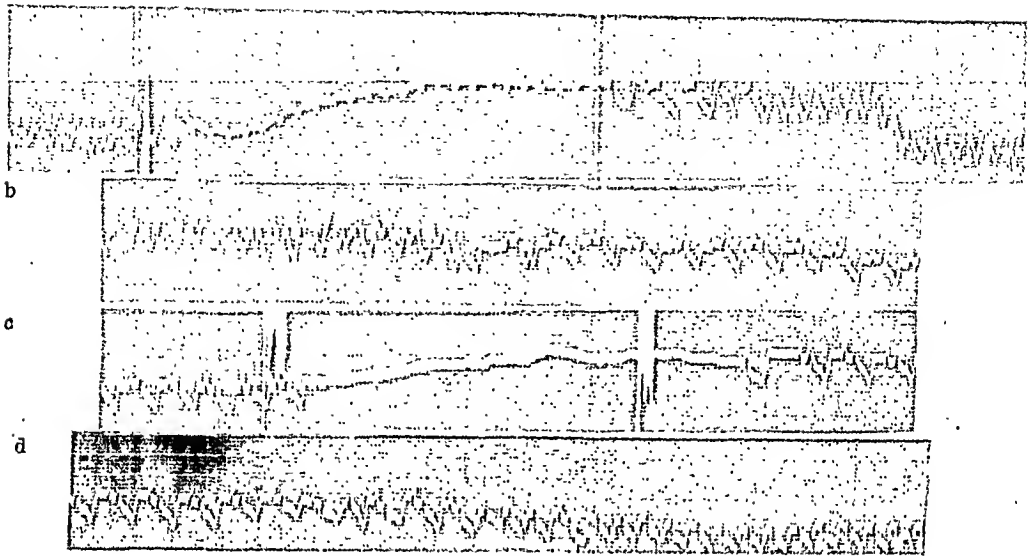


FIG. 3, a-d.

Vagus stimulation during auricular tachycardia (Fig. 3a), clamping off the focus in the right auricle (Fig. 3b), vagus stimulation during presence of clamp (Fig. 3c), and removal of clamp (Fig. 3d) (Lead II).

former tachycardia with a rate of 200 beats per minute.

Only rarely was the auricular tachycardia during vagus stimulation irregular. In Fig. 2b, which was secured in another experiment than Fig. 2a, at the beginning of the tracing the auricular rate after administration of aconitine was again 200. During stimulation of the right vagus nerve the rate increased to an average of 428 beats per minute and slight arrhythmias were visible. Temporary interruption of the contact of the stimulating electrode with the vagus nerve could not be excluded.

Fig. 3 is from an experiment in which the aconitine was injected on the tip of the right auricular appendix. A tachycardia with a rate of 272 appeared within a few seconds (Fig. 3a). Faradic stimulation of the right vagus led to an increase of rate to 375 beats per minute (Fig. 3a), and to a complete inhibition of the auriculo-ventricular conduction. The 2 perpendicular white lines represent signals showing the beginning and the end of the vagal stimulation. After stimulation was stopped a short period of 2:1 block

is noted (Fig. 3a), and the regular tachycardia with a rate of 280 recurred. Clamping off the right auricular appendix caused a sinus rhythm to appear immediately (Fig. 3b) with a rate of 150. In Fig. 3c, the effect of the vagus stimulation during the clamping, and the existence of a sinus rhythm is demonstrated; a complete standstill of the heart resulted, without any sign of activity in the electrocardiogram. Removal of the clamp permitted the tachycardia to recur immediately in the same form as before with a rate of 214 (Fig. 3d).

Fig. 4 was obtained in an experiment in which the injection was made on the appendix of the left auricle. A tachycardia with a rate of 310 appeared, and the rate increased during the vagus stimulation (see signals) only to 375 (Fig. 4a). Cooling of the site of origin of the tachycardia (the area of injection) led to the immediate disappearance of the tachycardia (Fig. 4b). A regular coronary sinus rhythm with a rate of 187 was present. Interruption of cooling was immediately followed by reappearance of the tachycardia, at the end of Fig. 4b. Usually

vagus stimulation not based on a circus movement mechanism is of interest.

Summary. Focal application of aconitine to the dog's auricle in the form of a subepicardial injection is followed by the appearance of a prolonged regular tachycardia with a rate of approximately 200 to 300 beats per minute. Faradic stimulation of the vagus nerves in the neck always leads to a remarkable increase of rate of the auricles. Auricular fibrillation or the phenomenon of rapid reexcitation were never observed during the vagus stimulation.

Separation of the site of injection from the rest of the heart by clamping abolishes the

tachycardia; it regularly reappears on removal of the clamp. Cooling of the site of injection by a thermode also stops the tachycardia, and it reappears immediately when cooling is discontinued.

The tachycardia shows characteristics of auricular flutter, but auricular tachycardia of the type "essential paroxysmal tachycardia" cannot be ruled out.

The results of the experiments cannot be explained under the assumption that the tachycardia is caused by a circus movement. Therefore the increase of rate during the vagus stimulation requires another explanation than the one given by Lewis.

15755

A Method for Intrathoracic Operation on the Rat.*

CHARLES B. PORTER AND JOHN T. SMALL. (Introduced by F. C. Mann.)

From the Division of Experimental Medicine, Mayo Foundation, Rochester, Minn.

Positive control of intrapulmonary tension and oxygenation is highly desirable for intrathoracic operations on any animal. The technic for larger animals, such as dogs, is well standardized, but because of the small size of the laboratory rat, none of the methods could be satisfactorily used in intrathoracic operations on that animal.

In connection with problems involving pneumotomy and pneumectomy in the rat, a simple and satisfactory method of positive pressure administration of oxygen has been evolved utilizing tracheal intubation. The rat is anesthetized with pentobarbital sodium intraperitoneally at a dosage of 30 mg per kg of body weight. In combination with this is given scopolamine hydrobromide, in a dosage of 0.6 mg for a 300 to 400 g rat.¹

* Work done in the laboratory of, and under the direction of, Dr. George M. Higgins, Division of Experimental Medicine, Mayo Foundation, Rochester, Minn.

¹ Hoke, H. G. O., Dosage of Drugs for Rats, in Griffith, J. Q., Jr., and Farris, E. J., *The Rat in Laboratory Investigation*, Philadelphia, J. B. Lippincott Company, 1942, chap. 13, pp. 297-350.

The latter drug was found to increase rapidly and depth of anesthesia, reduce tracheal secretions and counteract the respiratory depression that pentobarbital sodium tends to produce. The pharynx is cleared by suction and the trachea is intubated with a special tube to be described later. The end of the tube is then connected to the oxygen line and valve apparatus. Respiration is not artificial but is performed in the natural manner with the rat working against a variable pressure water valve.

The intratracheal tube is constructed of plastic tubing[†] having an outside diameter of 0.08 inch (0.20 cm) and an inside diameter of 0.045 inch (0.11 cm) (Fig. 1). One end is sealed off by rotating in a gentle flame and molding with the fingers, and a slight point is produced by grinding with an emery wheel. Just above the point 3 small holes, staggered around the circumference, are cut with fine scissors and ground smooth with

[†] Transflex, courtesy of the manufacturer, The Irvington Varnish and Insulator Company, Irvington, N.J.

unless the flutter rate before the stimulation is over 400 per minute.⁷ The gradual but rapid increase and decline of the auricular rate which has been observed in instances of auricular flutter during and after vagus stimulation⁴ is very similar to the phenomenon reported here.

In view of all these facts, it seems that a definite decision is impossible and the presence of either type of auricular tachycardia cannot be definitely ruled out.

A paradoxical increase in the formation of auricular and ventricular extrasystoles after vagus stimulation in the dog following intravenous injection of aconitine has been described as a regular phenomenon. Intravenous injection of choline preparations had the same effect.¹ The increase in the number of auricular extrasystoles in these experiments occurred however only *after* the stimulation of the vagus; during the stimulation only the usual inhibition of the auricles with standstill was seen.

The phenomenon of reexcitation and the marked increase of rate of the auricles, during vagus stimulation was used by Lewis and his associates in brilliant studies^{3,6,7} as one of the more important facts supporting the theory of circus movement. The shortening of the refractory phase during the stimulation of the vagus, which may amount to more than 1/5 of the normal, abolishes barriers in the form of islands of refractory muscle in the way of the circulation "Mother" wave and causes this wave to use a less sinuous path and therefore to circulate faster. Another possibility considered by Lewis would be the utilization of another path which is shorter than the original one around the orifices of the great veins.

One must concede that the circus movement theory explains both phenomena, the moderate increase of rate during vagus stimulation, as in the experiments reported here, and the appearance of the rates of over 3000 per minute, better than any other theory.⁸ A simple increase of the rate of stimulus formation is difficult to accept in view of the high rates which occur in rapid reexcitation

and in view of the known inhibitory action of the vagus on the heart. Rothberger and Winterberg, who explained flutter by a rapid stimulus formation in a single center, also tried to correlate the phenomenon of rapid reexcitation during vagus stimulation with a shortening of the refractory period; the finer mechanisms however are still unexplained. There is no information about the influence of vagus stimulation on the formation of stimuli in the centers, and it is possible that an increase of the rate of stimulus formation during vagus stimulation is a typical consequence of the shortening of the refractory period.

The observation that clamping off the site of origin of the tachycardia or cooling the point of injection of the aconitine abolished the tachycardia and immediately reintroduced sinus rhythm is of interest in connection with the 2 viewpoints regarding the mechanism of origin of auricular flutter. Still more important is the fact that interruption of the cooling causes the tachycardia immediately to reappear. These observations are not easily explained by a circus mechanism. It has been maintained that localized circus movements occur and cause localized auricular fibrillation which can be interrupted by clamping off or cooling a small area.⁹ The path of such a small circulating wave was estimated to use a circuit with a diameter of only 2.5 to 3.8 mm.⁷ If such a localized circus movement exists in these experiments however, it is hard to understand how interruption of the cooling is immediately followed by the reappearance of the same tachycardia as before. Without the assumption of a heterotopic focus of stimulus formation, this observation is difficult to explain.

Thus, in conclusion, one may state that if we are dealing with auricular flutter in these experiments, the effect of the cooling of the area into which the solution of aconitine was injected argues against the assumption of a circus movement. If we are dealing with a heterotopic tachycardia, the appearance of an acceleration of rate during

⁹ McWilliam, J. A., *Proc. Roy. Soc., London, Ser. B*, 1918, 90, 302.

⁸ Garrey, W. E., *Physiol. Rev.*, 1924, 4, 215.

partially collapsed lung may be obtained by briefly pinching off the rubber tubing leading to the water valve.

Oxygen flow is carefully regulated by means of the differential valve on the oxygen tank. When the flow is properly regulated, oxygen bubbles gently through the water valve at all times except during inspiration, when the flow is temporarily interrupted. Even during inspiration there should not be much rise of water level in the glass tubing of the valve.

For satisfactory operation, obstruction of the intratracheal tube must be avoided. If there is excessive trauma during intubation, blood is almost sure to clog the tube. Rats that have colds or mild chronic pneumonitis should not be used because excess secretion clogs the intratracheal tube. Suction or repeated insertion of the stylet will sometimes

free a clogged intratracheal tube.

We have used the method described in this paper in more than 300 thoracotomies on rats, about 200 of which were pneumonectomies. It has proved satisfactory and easy to use once the technic has been mastered. Best results were obtained when temperature and humidity were moderate. Hot, humid days seemed to cause a definite increase of the operative mortality rate.

Summary. For performance of intrathoracic operations on the rat pentobarbital sodium and scopolamine were found to be effective anesthetic agents. A plastic intratracheal tube was devised for use in such operations and has proved to be satisfactory. A simple water valve apparatus for administration of oxygen under positive pressure was devised for use with the intratracheal tube.

15756

Intravascular and Intracardiac Pressure Recording in Man: Electrical Apparatus Compared with the Hamilton Manometer.*

HURLEY L. MOTLEY, ANDRE COURNAND, LARS WERKO, DAVID DRESDALE, AARON HIMMELSTEIN AND DICKINSON W. RICHARDS, JR. (Introduced by W. W. Palmer.)

From the Department of Medicine, Columbia University, and the Chest and Medical Services of the Columbia University Division, Bellevue Hospital, New York City.

An electrical apparatus has been used for recording blood pressures, (peripheral artery, right auricle, right ventricle and pulmonary artery), pleural pressure, and mask pressure in man. The method was tested for accuracy and fidelity in reproduction of the pressure tracings by comparison with the Hamilton manometer¹ used extensively for the re-

cording of right heart pressure.^{2,3} Records were obtained of simultaneous tracings on both systems, permitting a beat to beat study (Fig. 1, A and B). A record was also taken on the Hamilton manometer with the electrical recording off (Fig. 1, C).

The electrical pressure pick-up unit used was the Clark Pressure Capsule.⁴ This capsule consists of 2 small coils mounted in a small round metal case (38 mm in diameter and 12.5 mm thick) and separated by a

* Under contract with Aero-Medical Laboratory, Wright Field, Dayton, Ohio. Additional support was provided by the Commonwealth Fund and the Life Insurance Medical Research Fund Gift for Study of Action of Certain Cardiovascular Drugs.

¹ Hamilton, W. F., Brewer, G., and Brotman, L. *Am. J. Physiol.*, 1934, **107**, 427.

² Cournand, A., Lauson, H. D., Bloomfield, R. A., Breed, E. S., and Baldwin, E. de F., *Proc. Soc. Exp. Biol. and Med.*, 1944, **55**, 34.

³ Bloomfield, R. A., Lauson, H. B., Cournand, A., Breed, E. S., and Richards, D. W., Jr., *J. Clin. Invest.*, 1946, **25**, 639.

⁴ Clark Capsule, designed by James C. Clark, Wright Field, Dayton, Ohio. Aerotronics Inc., Camden, Ohio, licensed to manufacture the capsule.

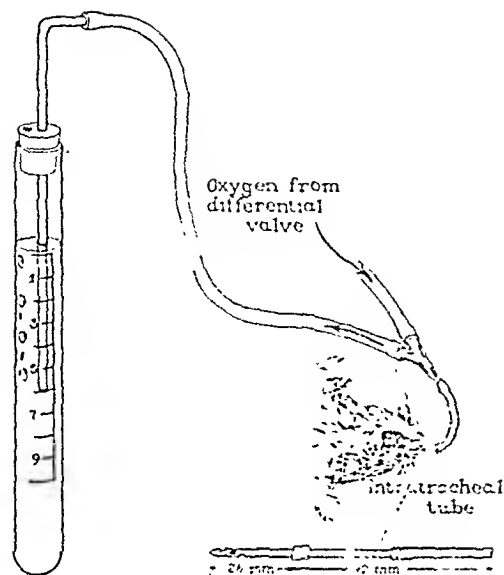


Fig. 1.

Positive pressure water valve, intratracheal tube and method of connection. The test tube is conveniently supported by a buret clamp attached to a ring stand. See text.

a small dental burr. A cuff of rubber tubing is placed with its lower edge 26 mm above the tip, in order to prevent insertion of the tube beyond the carina. A similar piece of tubing is slipped over the upper end of the plastic tube to serve as a connector and both are cemented in place with rubber cement. A stiff wire stylet is used during insertion.

Intubation must be done under direct vision in the rat owing to the extreme mobility of the laryngeal structures. A satisfactory "laryngoscope" can be a bivalve electric otoscope with half of the objective removed or an ordinary electric otoscope with the objective cut in half lengthwise (Fig. 2). The anesthetized rat is placed on its back on a rat board, the tongue is pulled well out, the upper jaw is held down by a loop over the front teeth and the tube is inserted between the vocal cords with the aid of the converted otoscope.

The positive pressure apparatus consists of a simple water valve (Fig. 1). A large test tube about 20 cm long is fitted with a 2-holed rubber stopper and filled about two-thirds

full of water. A glass tube about 6 mm in outside diameter slides through one of the holes in the stopper; the depth of immersion of the tube determines the amount of positive pressure. The second hole in the stopper allows escape of the excess gases. A centimeter scale marked on the side of the test tube allows exact settings. A glass Y tube completes the apparatus. One arm is connected to the differential valve on the oxygen tank, the second arm is connected to the water valve and the base of the Y is connected to the rubber tubing on the end of the intratracheal tube. It is very important to keep the last connection short in order to reduce the amount of physiologic dead space created by the intratracheal tube.

In using the apparatus a positive pressure of 1 to 2 cm of water is used until the thorax is about to be opened. For pneumonectomy or any operation in which maintaining a fully expanded lung is not important, the pressure is increased to 5 or 6 cm of water during the period when the thorax is open. If continued full expansion of the lung is desired, a pressure of 7 to 8 cm of water is necessary but this sometimes causes leakage of air into the mediastinum or the opposite pleural cavity, so that the lower pressures are generally more practical. Inflation of a

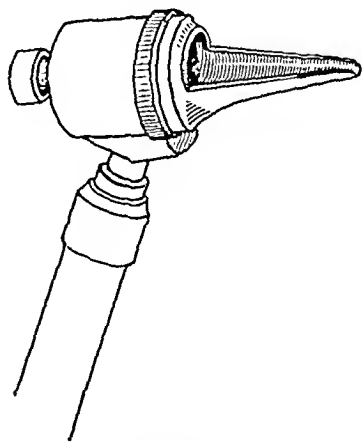


Fig. 2.

Converted electric otoscope used for the insertion of the intratracheal tube. Most of the top half of the objective has been cut away to form a miniature "laryngoscope."

idated Engineering Corporation, Serial No. 3341).

2. Amplifier (Consolidated Engineering Amplifier, 4-channel type 1-106, Serial No. 3123, and Oscillator, carrier type 2-104B, Consolidated Engineering, Serial No. 2983).

3. Recording camera (Oscillograph, recording, type 5-101A, Consolidated Engineering, Serial No. 4775).

By changing the amplification a wide range of pressures could be measured without going off scale. Large deflections of the tracings could be secured for any pressure range encountered, and the amplification adjustment could be made quickly by turning a dial as needed. The calibrations for the electric recordings were linear for any given amplification setting and capsule, and remained constant from day to day.

To record blood pressure, the Clark Capsule was filled with airless mineral oil on the side attached to a 3-way stopcock and connected to an arterial needle or a cardiac catheter, in order to maintain a continuous fluid system, (sodium citrate or saline solutions in the capsule produced enough electrolysis so that the balance of the bridge became unstable). The bridge was balanced and the zero lines recorded at the beginning and end of each tracing taken. This procedure prevents errors in base line which might be made due to voltage drop, resistance changes, or drifts. Usually, when the base line did change due to one of the above factors, the deflection was of small magnitude. The Clark Capsule can be attached very closely to either the intraarterial needle or the cardiac catheter without need for lead tubing.

A Sanborn Cardiette was attached to the recording oscillograph using the amplifier of the Cardiette and one of the galvanometers in the oscillograph, for simultaneous recording of the ECG with the pressure tracings. Tests on conduction as measured by simultaneous recording of a pressure wave and ECG impulse showed a lag of .005-.01 second for the pressure wave as compared to the ECG. Similar tests on the Hamilton apparatus showed a lag of .01 second.⁴

TABLE I.

		Hamilton	Electrical
Arterial mm Hg	Systolic	166.8	166.1
	Diastolic	108.2	107.7
	Mean	133.0	133.0
Right Ventricle mm Hg	Systolic	30.0	30.8
	Diastolic	6.96	7.12
	Mean	11.5	12.0
Pleural mm Hg	Max.	+1.6	+1.4
	Min.	-3.0	-2.0
	Mean	+0.5	+0.6
Mask mm Hg	Max.	9.0	10.0
	Min.	2.0	2.0
	Mean	4.0	5.0

Pressure tracings (brachial artery and pulmonary artery) and ECG lead II taken simultaneously on a cardiac case with left bundle branch block and an irregular heart beat, are shown in Fig. 1, D.

The pressures were carefully measured beat by beat as recorded by the electrical and Hamilton methods simultaneously for 12 consecutive corresponding heart beats as shown in Fig. 1, A and B. The 2 systems were connected for simultaneous recordings by the use of a modified 3-way stopcock. A comparison was also made of pleural and mask pressures on the 2 systems. Inspection of Fig. 1, A and B, shows the general shape of all tracings on the 2 systems to be quite similar. The average quantitative values for the 2 systems are shown in Table I.

When the electrical system was cut off there was no significant change in the Hamilton recording either in shape of tracing or pressure values, as shown in Fig. 1, C.

It is beyond the scope of this report to discuss whether the blood pressure tracings obtained through the cardiac catheter and intraarterial needle are true records of pressure variation within the heart cavity and large arteries. Pressure tracings whether recorded with the electrical pickup or a Hamilton type may be modified by (a) artifacts due to movement of the tip of the catheter or of the catheter itself within the heart, (b) small volume changes in the catheter

⁴ Courmand, A., Motley, H. L., Himmelstein, A., Dresdale, D., and Richards, D. W., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 148

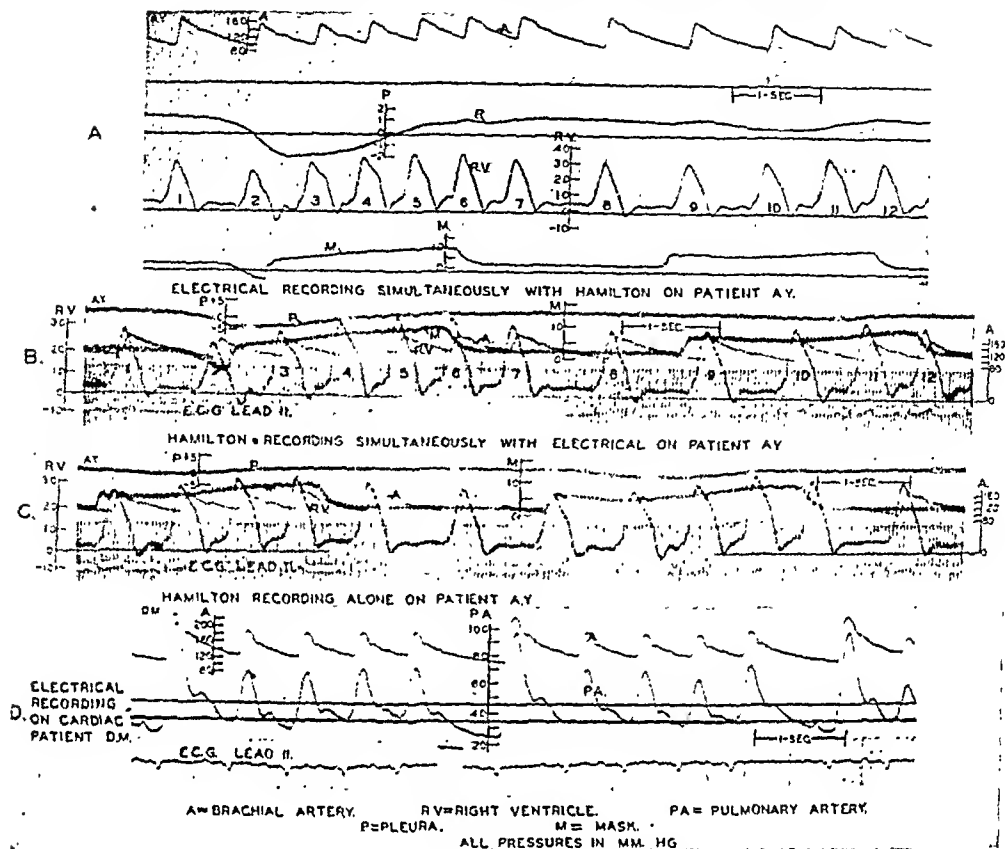


FIG. 1.

Electrical and Hamilton Pressure Recordings.

A. From above downward, brachial artery pressure, intra-pleural pressure, right ventricle pressure and face mask pressure. Patient breathing with an intermittent positive pressure respirator. Artificial pneumothorax previously present connected for pressure recording. Electrical recordings made simultaneously with the Hamilton given below and 12 consecutive corresponding heart beats shown, both arterial and right ventricle pressure pulse waves, the latter being numbered for ease in identification.

B. From above downward, intra-pleural pressure, face mask pressure, brachial artery pressure, right ventricle pressure and ECG lead II. Hamilton manometer recording made simultaneously with the electrical recording in A on the same patient.

C. Hamilton manometer recording alone on the same patient as above, but with the electrical apparatus disconnected. Tracing taken just previous to A and B shown above. Pressure tracings and ECG in the same order as in B above.

D. Electrical recording of brachial artery pressure (upper) and pulmonary artery (lower) with simultaneous ECG lead II in a cardiac patient with both systemic and pulmonary hypertension and a left bundle branch block.

diaphragm of varying thickness, depending on the pressure sensitivity required. The coils carrying a 1000 kc current on each side of the diaphragm of the capsule are balanced in the circuit of a bridge. When the 2 coils are properly balanced in the bridge a galvanometer connected through an amplifier for recording is set on the camera screen at a desired zero point. When pressure is ex-

erted on one side of the capsule, a slight movement of the diaphragm occurs which alters the magnetic reluctance, creating an electrical imbalance of the bridge, which is amplified and the resulting galvanometer deflection recorded on an oscillograph type recording camera.

Electrical parts used:

1. Bridge (Balance type 3-103B Consol-

idated Engineering Corporation, Serial No. 3341).

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⁴ Cournaud, A., Motley, H. L., Himmelstein, A., Dresdale, D., and Richards, D. W., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 148

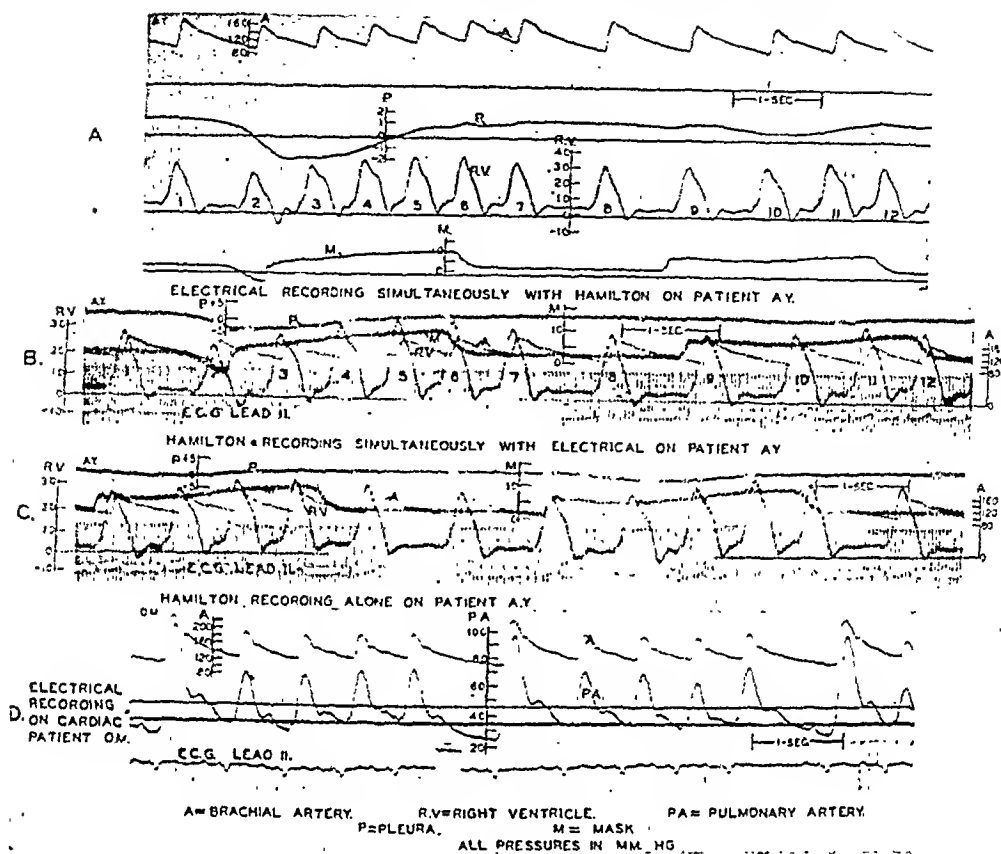


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diaphragm of varying thickness, depending on the pressure sensitivity required. The coils carrying a 1000 kc current on each side of the diaphragm of the capsule are balanced in the circuit of a bridge. When the 2 coils are properly balanced in the bridge a galvanometer connected through an amplifier for recording is set on the camera screen at a desired zero point. When pressure is ex-

erted on one side of the capsule, a slight movement of the diaphragm occurs which alters the magnetic reluctance, creating an electrical imbalance of the bridge, which is amplified and the resulting galvanometer deflection recorded on an oscillograph type recording camera.

Electrical parts used:

1. Bridge (Balance type 3-103B Consol-

Swabs and membrane of the tonsillar ulcer of each patient, together with his saliva, were rubbed onto the scarified cornea of a rabbit. None of the 12 rabbits developed the acute keratoconjunctivitis characteristic of an infection with the virus of Herpes Simplex,¹ or survived a challenge dose of this virus when given intracerebrally after an interval of about 3 weeks. Neutralization tests done with early and convalescent sera of these patients against a standard (HF) strain of herpes showed that those patients with absence of antibodies during the disease did not develop them in convalescence, while those with neutralizing antibodies in the acute serum did not show a rise of titer in the convalescent serum.

Technics. Those used for virus and for antibody studies were identical with standard methods as used by the authors in a previous study.¹

Discussion. It has been shown that the common form of acute infectious gingivostomatitis as seen most frequently in children and occasionally in adults is due to a primary infection with the virus of Herpes Simplex. Since the etiology of this disease was in the past frequently ascribed to Vincent's organ-

isms, although it is now known that it is caused by the virus of Herpes Simplex, a study was undertaken to determine whether the so-called Vincent's angina of the tonsil was also caused by this virus with the spirochete-fusiform combination acting as secondary invaders. In the 12 patients with Vincent's angina of the tonsil here studied, neither was the virus demonstrated in the membrane or saliva, nor did neutralizing antibodies against Herpes Simplex virus appear during convalescence. These facts, together with the striking effect of penicillin² in this disease, would negate any etiologic role of Herpes Simplex virus and is suggestive of a primary etiological role of the spirochetes themselves.

Summary. A study of 12 typical cases of Vincent's tonsillar angina showed that none of the cases were caused by an underlying infection with the virus of Herpes Simplex.

2 a. Denny, E. R., Stallenberger, P. H., and Pyle, H. D., *J. Oklahoma M. A.*, 1944, 37, 193; b. Naegeli, F. C., and Morginson, W. J., *J. Am. Dental Assn.*, 1945, 32, 1393; c. Shellenberger, P. L., Denny, E. R., and Pyle, H. D., *J. A. M. A.*, 1945, 128, 706; d. Schwartz, B. M., *J. A. M. A.*, 1945, 128, 704.

15758

Effects of Some Old and Proposed Anticonvulsants on the Threshold for Electrical Convulsions.

WEI-CHANG CHU AND ROBERT L. DRIVER.

From the Department of Pharmacology and Therapeutics, Stanford University School of Medicine, San Francisco, Calif.

Desirable improvements on diphenylhydantoin and other anticonvulsants in the treatment of epilepsy have stimulated the investigation of a large number of compounds with the hope of finding some agent which would prevent or mitigate epileptiform seizures without undesirable side effects.

Method. One of the methods used in these studies has been the production of epileptiform convulsions by passing an electrical current of varying amperage through the brains of animals. The threshold in

milliamperes (m.a.) for each animal is determined before (control) and after the administration of the drug to be tested, the difference being considered a measure of the effectiveness of the drug as an anticonvulsant. The method has been described in detail by Spiegel,¹ Putnam and Merritt² and Tainter

¹ Spiegel, E. A., *J. Lab. and Clin. Med.*, 1937, 22, 1274.

² Putnam, T. J., and Merritt, H. H., *Science*, 1937, 85, 525.

due to its deformability, and (c) low damping frequency of the entire hydraulic system ranging from 12 to 40 cycles per second. The volume change in the Clark Capsule is slightly larger than in the Hamilton type of capsule, for a given pressure alteration. This might affect slightly the form of the pressure tracing. In so far as can be determined by comparison with the Hamilton, however, the Clark Pressure Capsule appears to be adequate for the purpose of measuring systolic, diastolic and mean blood pressures (Fig. 1).

The advantages of the electrical recording device are: (a) the indefinite use of the capsule membrane, (b) the constancy of the linear calibrations, (c) the ease of rapid adjustment of the amplification in order to meet any pressure range, as required by displacement of the catheter from right ventricle to the right auricle, etc., (d) the absence of lead tubing, (e) the more rapid recordings and (f) better control of the base lines. The

disadvantages are: (a) the slightly larger volume change required in the Clark Pressure Capsule per unit of pressure change, as indicated above, (b) the high cost of the electrical amplification equipment as used in this study. The apparatus as described here for amplification has a much wider range than is necessary for blood pressure recordings. By further modification of this type apparatus, to meet the special needs of blood pressure recording, it should be possible to overcome these disadvantages.

Summary. 1. The Clark Capsule, an electrical pressure pickup device, has been found satisfactory for recording blood pressures from the right heart, pulmonary artery, systemic arteries, pleural and mask pressures in man, when used with a suitable amplification and recording device.

2. The tracings recorded with this apparatus compare closely with those obtained simultaneously with a Hamilton manometer system.

15757

Evidence that Virus of Herpes Simplex Does Not Cause Vincent's Angina of the Tonsil.*

ALEX J. STEIGMAN† AND THOMAS F. MCNAIR SCOTT.‡ (Introduced by W. Henle.)

The etiological role of the virus of Herpes Simplex in acute infectious gingivostomatitis has been established.¹ Not infrequently the Plaut-Vincent spirochete and the fusiform bacilli are also present, perhaps as secondary

invaders, and prior to proof that the virus of Herpes Simplex was responsible, the spirochete-fusiform bacilli were regarded by some as causal. Thus, the disease was often called Vincent's gingivostomatitis.

It seemed worthwhile to investigate whether or not the virus of Herpes Simplex had any underlying causal role in Vincent's tonsillar angina. Each patient studied had a typical unilateral, craggy, yellowish ulcerated tonsil with membrane and foul odor. There was local adenopathy and low, if any, fever. Many Vincent's spirochetes and fusiform bacilli were seen on smear; no β -hemolytic streptococci or *C. diphtheriae* were cultured and no evidence of syphilis or infectious mononucleosis demonstrated.

Twelve young British soldiers (18 to 35 years of age) had the above typical picture.

* Work done while serving with the American Red Cross Field Hospital Unit, Salisbury, England, 1941-1942.

† Department of Pediatrics, Temple University Medical School, Philadelphia, Pa.

‡ The Children's Hospital of Philadelphia (Department of Pediatrics, University of Pennsylvania), Philadelphia, Pa.

1a. Dodd, Katherine, Buddingh, John, and Johnston, Leland, *Am. J. Dis. Child.*, 1939, **58**, 907; b. Burnet, F. M., and Williams, S. W., *M. J. Australia*, 1939, **1**, 637; c. Scott, T. F. McNair, and Steigman, Alex J., *J. A. M. A.*, 1941, **117**, 999; d. Black, W. C., *J. Pediat.*, 1942, **20**, 145.

TABLE I.
Effect of Various Agents on Thresholds for Electrical Convulsions.

Compound	No. of rats	Dose, mg/kg	Route of administration*	Mean change in threshold, m.a.f	S.E. of mean, m.a.f	Avg incr. in threshold, %	Remarks
Demerol	20	5-25	II	+0.6	± 0.27	7	No or slight motor depression
"	4	50	II	+2.0	± 0.55	28	Coma, fatal
Propazone	8	130-250	IP	+3.2	± 0.53	43	Slight ataxia to coma
Diphenylhydantoin	5	46	G	0	± 0.25	0	Asymptomatic
"	5	40	IV	+1.0	± 0.50	10	"
"	4	100	IV	+3.3	± 0.60	41	Slight motor depression
3-indolyl methylene hydantoin	14	100-400	IM	+0.4	± 0.42	40	Asymptomatic
Methyl-N-hexyl hydantoin	5†	25	IV & IM	0	± 0.32	0	"
"	5	50	IV & IM	0	± 0.44	0	Motor depression
"	5	100	IV & IM	+1.2	± 0.82	15	"
"	10	200	IV & IM	+4.4	± 1.45	55	"
Methyl-N-amyl hydantoin	5†	50	II	0	± 0	0	Narcosis
"	5	50	G	+1.6	± 0.71	20	Asymptomatic
"	5	100	G	+2.4	± 0.58	34	Slight ataxia
"	5	160	G	+4.4	± 0.87	52	"
"	5	50	IM	+2.0	± 0	25	"
"	4	80	IM	+3.0	± 0.55	37	Asymptomatic
"	5	160	IM	+7.6	± 0.70	95	Slight ataxia
Di-isobutyl hydantoin	5	50	IM	+2.0	± 0	28	Moderate ataxia
"	5	100	IM	+1.2	± 1.00	15	Asymptomatic
"	5	160	IM	+6.4	± 0.75	80	Slight ataxia

* G—gastrointestally; II—hypodermically; IP—intraperitoneally; IM—intramuscularly; IV—intravenously.

† Millamperes.

‡ Rabbits.

and associates.³ We used the technical arrangement described by Tainter and associates.³

In this arrangement, a high resistance stimulator and a 110-volt, 60-cycle, alternating current are used. In making a determination of cortical threshold, the transformer is set to give the voltage required for the desired m.a. of current, the electrodes (clamps) are clipped on the ears of the rats, and the stimulus is applied for exactly 10 seconds. In rabbits one electrode is applied to a bit in the mouth, and the other electrode to a small sponge-rubber pad resting on the clipped occipital skin moistened with saline solution. With currents below the threshold level there are generalized muscular contractions, synchronous with the current (tonic phase). At or above the threshold levels the tonic phase passes gradually into the clonic or epileptiform phase. The stimulation in rats is usually begun with a current of 6 m.a. (in rabbits, 14 m.a.), and is repeated at 5-minute intervals increasing the amount 2 m.a. each time until the threshold value is reached. This value remains practically unchanged for the purpose at hand, since it is reproducible within 1 to 2 m.a. when determinations are repeated at intervals of several days. Frequent, *e.g.*, consecutive daily, stimulations, however, tend to produce higher thresholds. Therefore, the animals are always allowed to rest 3 to 5 days before a drug is administered and the threshold redetermined. This procedure has been used routinely for several years in this department on hundreds of animals and with numerous drugs, and therefore, results, even with small groups of animals, are of significance in screening tests.

Employing this technic, a number of old agents and some proposed anticonvulsants were studied and the findings are reported in this paper. The test animals were chiefly adult white rats (total 185) with control thresholds ranging from 6 to 16 m.a. (aver-

age, 8 m.a.). A few rabbits (total 25; 3 to a drug) were used for intravenous injections. Their thresholds usually were higher than in rats, ranging from 14 to 28 m.a. (average, 18 m.a.), although the results with the drugs used were similar to those obtained in rats and are described together. The drugs were given by various routes $\frac{1}{2}$ to 1 hour before redetermination of the threshold, a change in which was not considered significant when, because of individual variations, it did not exceed the control threshold by more than 10%. When drugs were given gastrically, food was withdrawn from those animals one day previously. In the majority of tests, 5 rats were used for each drug and each dose, and occasionally up to 20 were used. The results obtained in rats with compounds which gave positive anticonvulsant activity are given in Table I.

Positive Agents. Demerol (isonipecaine, meperidine or pethidine) and propazone⁴ were found to be effective only in excessive doses, as was methyl-N-hexylhydantoin (Table I). Of the other new hydantoins* studied, only 2, namely, methyl-N-amylhydantoin and di-isobutyl-hydantoin, showed any promise, and of these the former was effective only in doses which caused slight but definite ataxia. There was less ataxia with higher doses of di-isobutyl-hydantoin, but there was more variability, suggesting that cortical excitability did not decrease proportionately with increase in dosage of the drug. In general, this drug seemed somewhat comparable to diphenylhydantoin.

Negative Agents. Tests were also made with the following drugs of the curare group: curare (2.5 to 5.0 mg per kg intramuscularly), β -erythroidine (10 to 400 mg per kg hypodermically; 50 to 800 mg per kg gastrically), dihydro- β -erythroidine (10 to 100 mg per kg hypodermically; 10 to 30 mg per kg intravenously) and quinine ethochloride (4 to 100 mg per kg hypodermically). These

³ Tainter, M. L., Tainter, E. G., Lawrence, W. S., Neuru, E. N., Laekey, R. W., Ludueña, F. P., Kirtland, H. B., and Gonzales, R. I., *J. Pharm. and Exp. Therap.*, 1943, **79**, 42.

⁴ Luton, F. H., Blalock, J., Baxter, J. H., Jr., and Stoughton, R. W., *Proc. Soc. Exp. Biol. and Med.*, 1941, **47**, 245.

* Obtained from Dr. Melville Sahyun, Frederick Stearns & Co., Detroit, Mich.

drugs did not raise the threshold, except in excessive doses which caused incomplete paralysis, or convulsions. With asymptomatic doses thresholds were unchanged or even decreased (possible excitatory action of anoxemia). These results did not support the claim that the curare group of drugs exert a central depressant action.⁵

The beneficial effects of certain dyes on patients, reported by different authors,⁶ suggested studies of these agents, but trials with hematoxylin (1.5 g per kg), congo red and vital red (each 50 mg per kg intravenously), neutral red, phenol red and methyl red (each 50 mg per kg intraperitoneally) resulted in no change in threshold.

Other agents which were found to be without anticonvulsant activity were ammonium thiocyanate (0.1 g per kg gastrically), magnesium sulfate (0.7 g per kg intraperitoneally), theophylline sodium acetate (50 mg per kg hypodermically) and glutamic acid (0.1 to 0.2 g per kg hypodermically). Voluntary drinking of dilute acid (0.5% HCl) and of dilute alkali (1% NaHCO₃ or 0.01% NaOH) for 6 days, in place of drinking water, also proved ineffective.

A decrease in threshold of about 30% was obtained by depriving rats for 6 days of either water or food or both.

Summary. Over 400 tests were made in 210 animals with 23 different agents on the threshold for electrical convulsions, chiefly in white rats; a few in rabbits. Of 3 new hydantoins studied, di-isobutyl-hydantoin was the most promising, being somewhat comparable to sodium diphenylhydantoin in high doses. Among the ineffective agents were 4 drugs of the curare group, alleged central depressants, and 5 different dyes, including hematoxylin and vital red, alleged antiepileptic agents.

15759

Isopropyl Alcohol, Other Ketogens, and Miscellaneous Agents on Thresholds for Electrical Convulsions and Diphenylhydantoin

ROBERT L. DRIVER.

Department of Pharmacology and Therapeutics, Stanford University School of Medicine, San Francisco, Calif.

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summary of the essential features of this method, adapted to rats, is given in a previous paper by Chu and Driver,² and need not be repeated here.

The control threshold, which ranged from 6 to 16 milliamperes (m.a.) (average, 8 m.a.),

¹ Tainter, M. L., Tainter, E. G., Lawrence, W. S., Neurn, E. N., Lackey, R. W., Ludueña, F. P., Kirtland, H. B., and Gonzales, R. I., *J. Pharm. and exp. Therap.*, 1943, 79, 42.

Chu, W. C., and Driver, R. L., *Proc. Soc. Exp. and Med.*, 1947, 64, 245.

TABLE I.
Isopropyl Alcohol and Ketogens on Thresholds for Electrical Convulsions and Diphenylhydantoin.

Compound*	No. of rats	Dose, mg/kg	Route of administration†	Hr before test	Mean change in threshold, m.a.‡	S.E. of mean, m.a.‡	Avg incr. in threshold, %
Acetone	5	1250	G	1	+ 4.4	±0.80	46
"	5	"	G	4	+ 1.2	±0.55	14
Acetophenone	3‡	"	G	1	+ 7.3	±0.60	87
Ethyl acetacetate	5	"	G	1	+ 1.6	±0.75	15
Methyl alcohol	5	"	G	1	+ 4.4	±0.82	41
Ethyl "	5	"	G	1	+ 2.8	±0.80	26
Propyl "	5	"	G	1	+ 3.2	±1.10	26
Isopropyl "	5	"	G	1	+ 7.2	±0.85	82
" "	5	"	G	2	+ 4.8	±0.62	57
" "	4	"	G	4	+ 7.0	±1.40	75
" bromide	5	"	G	1	+ 2.8	±0.87	32
Diacetone alcohol	5	"	G	1	+ 4.8	±0.51	55
" glycol	5	"	G	1	+ 6.0	±0.65	71
Sodium diphenylhydantoin	5	40	G	1	+ 0	±0.25	0
" "	5	150	G	2	+ 1.2	±0.53	13
" "	5	40	IV	1	+ 1.0	±0.50	10
" "	4	100	IV	1	+ 3.3	±0.60	41
" "	4	40	IM	2	+ 0	±0	0
" "	5	100	IM	2	+ 1.0	±0.45	11
" "	5	150	IM	2	+ 2.0	±0.70	23
" "	5	40	G	1	+ 4.4	±0.42	40
+ ethyl alcohol		1250	G	1			
Sodium diphenylhydantoin	5	40	G	1	+ 12.2	±2.02	115
+ isopropyl alcohol		1250	G	1			

* An insoluble compound was administered as a suspension or emulsion.

† G—gastrically; IV—intravenously; IM—intramuscularly.

‡ 2 rats died.

§ Milliampères.

was established for each rat at least 4 days before testing the effect of a compound. The substance was administered at a given time before the test, and the threshold redetermined. The difference in the thresholds so obtained was taken as a measure of the effectiveness of the medication, and, because of individual variations, had to exceed 10% to be considered significant. Food was withdrawn from all animals the night before the day of the test. The results (average % changes) including the standard errors (S.E.), with a number of positive agents and combinations are given in Table I. A total of 265 rats was used, in groups of 5 for each agent or combination in the majority of tests.

Isopropyl Alcohol and Other Ketogens. The results in Table I indicate that a number of compounds excelled sodium diphenylhydantoin as an anticonvulsant. Of particular interest were the compounds closely related to acetone in chemical structure. Of these, isopropyl alcohol, which exerted a tre-

mendous anticonvulsant effect without ataxia or narcosis, proved to be one of the most promising. The dosage used, i.e., 1250 mg per kg, was only about one-fourth the anesthetic dose. The animals were somewhat quieter than normals, but their reflexes and voluntary locomotion were preserved. Three months later all were alive and in good condition. The safeness of this agent for oral use has been reported by Harris.^{3,4} Boughton,⁵ comparing the relative toxicity of ethyl alcohol and isopropyl alcohol in rats given 5% solutions of these alcohols for 9 months, concluded that isopropyl was only slightly more toxic than ethyl alcohol. Lehman and Chase,⁶ studying the acute and chronic tox-

³ Harris, L. E., *Drug and Cosmetic Ind.*, 1944, **51**, 44.

⁴ Harris, L. E., *J. Am. Pharmaceutical Assn., Pract. Pharm. Ed.*, 1944, **5**, 38.

⁵ Boughton, L. L., *J. Am. Pharmaceutical Assn.*, 1944, **33**, 111.

⁶ Lehman, A. J., and Chase, H. F., *J. Lab. and Clin. Med.*, 1944, **29**, 561.

drugs did not raise the threshold, except in excessive doses which caused incomplete paralysis, or convulsions. With asymptomatic doses thresholds were unchanged or even decreased (possible excitatory action of anoxemia). These results did not support the claim that the curare group of drugs exert a central depressant action.⁵

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⁵ Culler, E. A., *Proc. Am. Physiol. Soc.*, 1939, p. 56; Feitelberg, S., and Pick, E. P., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 654; Harvey, A. M., and Masland, R. L., *J. Pharm. and Exp. Therap.*, 1941, **73**, 304; Pick, E. P., and Unna, K., *J. Pharm. and Exp. Therap.*, 1945, **83**, 59.

⁶ Cobb, S., and Cohen, M. E., *Arch. Neurol. Psychiat.*, 1938, **40**, 1156; Osgood, R., and Robinson, L. J., *Arch. Neurol. Psychiat.*, 1938, **40**, 1178; Aird, R. B., *Arch. Neurol. Psychiat.*, 1939, **42**, 700.

Other agents which were found to be without anticonvulsant activity were ammonium thiocyanate (0.1 g per kg gastrically), magnesium sulfate (0.7 g per kg intraperitoneally), theophylline sodium acetate (50 mg per kg hypodermically) and glutamic acid (0.1 to 0.2 g per kg hypodermically). Voluntary drinking of dilute acid (0.5% HCl) and of dilute alkali (1% NaHCO₃ or 0.01% NaOH) for 6 days, in place of drinking water, also proved ineffective.

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The efficacy but impracticability of a ketogenic diet in the treatment of patients subjected to epileptic seizures suggested the use of products of fat and carbohydrate metabolism and related compounds. With this in mind, the following experiments were carried out.

Method. Clonic (epileptiform) convulsions were produced by passing an electric current for 10 seconds through the brains of white rats, according to a technic described in detail by Tainter and associates.¹ A

summary of the essential features of this method, adapted to rats, is given in a previous paper by Chu and Driver,² and need not be repeated here.

The control threshold, which ranged from 6 to 16 milliamperes (m.a.) (average, 8 m.a.),

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² Chu, W. C., and Driver, R. L., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 245.

(or isopropanol) for benefits from ketogenic agents in epilepsy and for developing new agents or improving old antiepileptic agents is discussed.

15760

Hydropic Changes in Pancreatic Ductules and Islets in Alloxan Diabetes in the Rabbit.*

G. LYMAN DUFF, GARDNER C. McMILLAN,[†] AND DONALD C. WILSON.
(Introduced by J. B. Collip.)

From the Department of Pathology, Pathological Institute, McGill University.

Several investigators have described hydropic degeneration of the pancreatic islets and ductules in dogs rendered diabetic by partial pancreatectomy^{1,2} and by anterior pituitary extracts.³⁻⁵ In dogs made diabetic by alloxan, hydropic degeneration of islet cells has not been observed even in the presence of extreme vacuolation of the epithelium of the intralobular pancreatic ducts.⁶ Hydropic changes have been observed in the islets of cats that had become diabetic following partial pancreatectomy² or treatment with anterior pituitary extract⁷ but the pancreatic ductules were not affected. While such alterations in occasional islet cells have been described in rabbits treated with al-

loxan^{8,9} the duct epithelium is said to remain normal.¹⁰ The only description of alterations in the epithelium of pancreatic ductules in diabetic rabbits is that of Ogilvie¹¹ who found slight vacuolation of the lining cells of "newly formed" intralobular ducts in 1 of 28 rabbits treated with anterior pituitary extract. It is interesting, therefore, to report a high incidence of moderate to extreme hydropic degeneration of both the ductules and islets of the rabbit's pancreas following diabetes of long duration induced by alloxan.

Materials and Methods. Each of 56 white domestic rabbits obtained from several different dealers received intravenously 200 mg of alloxan (Eastman) per kg of body weight in a 5% aqueous solution. They were treated with protamine zinc insulin and glucose for a period of not more than 14 days following alloxan injection. In all of these animals and in a control group of 26 untreated rabbits, repeated determinations were made of fasting blood sugar and urinary sugar and acetone. Surviving animals of the experimental group were killed with corresponding control animals at various intervals up to one year after injection of alloxan.

Observations. Of the 56 rabbits treated with alloxan, 53 became persistently diabetic

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[†] Medical Research Fellow of the National Research Council, Canada.

¹ Allen, F. M., *Studies Concerning Glycosuria and Diabetes*, Cambridge, Mass., Harvard Univ. Press, 1913; *J. Metab. Res.*, 1922, 1, 5.

² Homans, J., *Proc. Roy. Soc., London, Ser. B*, 1913, 86, 73; *J. Med. Res.*, 1914, 30, 49; *J. Med. Res.*, 1915, 33, 1.

³ Richardson, K. C., *Proc. Roy. Soc., London, Ser. B*, 1939-40, 128, 153.

⁴ Ham, A. W., and Haist, R. E., *Am. J. Path.*, 1941, 17, 787.

⁵ Dokan, F. C., Fish, C. A., and Lukeus, F. D. W., *Endocrinology*, 1941, 28, 341 b.

⁶ Goldner, M. G., and Gomori, G., *Endocrinology*, 1943, 33, 297.

⁷ Lukeus, F. D. W., and Dokan, F. C., *Endocrinology*, 1942, 30, 175.

⁸ Bailey, O. T., Bailey, C. C., and Hagan, W. H., *Am. J. Med. Sc.*, 1944, 208, 450.

⁹ Kennedy, W. B., and Lukeus, F. D. W., *Proc. Soc. Exp. Biol. and Med.*, 1944, 57, 143.

¹⁰ Duffy, E., *J. Path. and Bact.*, 1945, 57, 199.

¹¹ Ogilvie, R. F., *J. Path. and Bact.*, 1944, 56, 225.

icity of isopropyl alcohol given to, and drunk voluntarily by rats reported no evidence of delayed toxic effects and no suggestion of harmful intermediate products, while the acute effects were similar to those of ethyl alcohol. These reports are typical of many on the toxicity of isopropyl alcohol. Its superiority to ethyl alcohol as an anticonvulsant is, according to biochemical evidence, probably due to its partial transformation to acetone in the body.⁷⁻¹¹ This may also be the explanation of the beneficial effects of a ketogenic diet in epileptics, although, of course, the ultimate mechanism remains unsolved.

Beta-hydroxy-butyric acid and acetoacetic acid were not tried, but diacetone alcohol* (4-hydroxy-2-keto-4-methylpentane) and diacetone glycol* (2,4-dihydroxy-4-methylpentane) were quite effective, while ethyl acetoacetate was slightly so. The difference in activity between isopropyl alcohol and acetone was possibly due to differences in rate of absorption and excretion.⁷ Acetone, after 4 hours, caused only a small increase in the threshold, presumably because this agent was rapidly eliminated from the body due to its volatility. The positive effect of isopropyl bromide could conceivably have been due to 2 things: (1) liberation of bromide and (2) formation of acetone. Acetophenone was quite effective but was too toxic because it caused fatalities. Aside from acetophenone, in this group of ketogens, only diacetone alcohol caused demonstrable ataxia.

Isopropyl Alcohol and Diphenylhydantoin Combined. Since the available evidence favored isopropyl alcohol as the least hazardous and most desirable of all the agents tested,

it was tried in combination with diphenylhydantoin. A greater effect than summation of activity, perhaps a sensitization, was obtained by combining sodium diphenylhydantoin in doses of 40 mg per kg with isopropyl alcohol, given gastrically, because diphenylhydantoin itself caused no significant increase in threshold, while the combination of the 2 drugs exceeded considerably the depression caused by isopropyl alcohol itself (Table I). Ethyl alcohol, combined the same way, only moderately exceeded the threshold for the alcohol itself, thus indicating that isopropyl alcohol was more specific in this respect. Here again, raising of the cortical threshold with the combinations of isopropyl alcohol and diphenylhydantoin occurred without demonstrable evidence of ataxia or narcosis, and all the animals recovered. It is suggested that the isopropyl group (or isopropanol), in view of its relative nontoxicity and close correlation with the ketogenic mechanism, would seem to offer possibilities for developing new, or improving old, anti-epileptic agents.

Ineffective Agents. The following compounds were found to be ineffective: the sugars—glucose, fructose, galactose, sucrose and triose (each 1.5 g per kg); the organic acids—lactic, pyruvic, acetic, propionic, butyric, palmitic, citric, malic, succinic, malonic and fumaric (each 0.5 g per kg); iodoacetic acid (0.02 g per kg); the alcohols—butyl, isobutyl and isoamyl (each 1.25 g per kg); insulin (2.5 units per kg), isopropyl ether and ethyl acetate (each 1.25 g per kg). All these agents were given gastrically, except insulin and iodoacetic acid, which were injected hypodermically.

Summary. The majority of certain compounds tried, and related in chemical structure to acetone, particularly isopropyl alcohol, were found to raise considerably the threshold for electrical convulsions in rats. Isopropyl alcohol increased the cortical depressant efficiency of diphenylhydantoin without demonstrable motor depression or narcosis. Some other alcohols and certain sugars and organic acids were ineffective. The possible significance of the isopropyl group

⁷ Kemal, H., *Biochem. Z.*, 1927, **187**, 461.

⁸ Kemal, H., *Z. physiol. Chem.*, 1937, **246**, 59.

⁹ Neymark, M., *Scand. Arch. Physiol.*, 1938, **78**, 242.

¹⁰ Morris, H. J., and Lightbody, H. D., *J. Ind. Hyg. and Tox.*, 1938, **20**, 428.

¹¹ Lehman, A. J., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 232.

* These and some other related agents were supplied by the Shell Development Co., Emeryville, Calif.

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² Homans, J., *Proc. Roy. Soc., London, Ser. B*, 1913, **86**, 73; *J. Med. Res.*, 1914, **30**, 49; *J. Med. Res.*, 1915, **33**, 1.

³ Richardson, K. C., *Proc. Roy. Soc., London, Ser. B*, 1939-40, **128**, 153.

⁴ Ham, A. W., and Haist, R. E., *Am. J. Path.*, 1941, **17**, 787.

⁵ Dohan, F. C., Fish, C. A., and Lukens, F. D. W., *Endocrinology*, 1941, **28**, 341 b.

⁶ Goldner, M. G., and Gomori, G., *Endocrinology*, 1943, **33**, 297.

⁷ Lukens, F. D. W., and Dohan, F. C., *Endocrinology*, 1942, **30**, 175.

⁸ Bailey, O. T., Bailey, C. C., and Hagan, W. H., *Am. J. Med. Sci.*, 1944, **208**, 450.

⁹ Kennedy, W. B., and Lukens, F. D. W., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 143.

¹⁰ Duffy, E., *J. Path. and Bact.*, 1945, **57**, 199.

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HYDROPIC CHANGES IN DIABETIC RABBITS

TABLE I.
Summary of Experimental Data.

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Duration of diabetes in mo.	No. of animals	Grade of Hydropic Degeneration										Mean of avg blood sugar mg per 100 cc
		Islets					Ductules					
		0	1	2	3	4	0	1	2	3	4	
0-1	19	19	—	—	—	—	19	—	—	—	—	428
1-2	8	1	2	4	1	—	1	3	2	—	2	388
2-3	4	1*	1	2	—	—	—	—	2	—	2	379
3-4	9	1†	2	4	—	2	1†	—	—	3	5	422
4-5	4	—	—	2	2	—	—	—	1	1	2	432
5-6	2	1‡	—	1	—	—	1‡	—	1	—	—	310
6-7	2	—	—	1	1	—	—	—	1	—	—	337
7-8	2	—	—	2	—	—	—	—	1	1	—	444
8-9	2	—	—	1	1	—	—	—	1	1	—	401
12	1	—	—	—	1	—	—	—	—	1	—	256
Totals	53	23	5	17	6	2	22	3	9	7	12	
Control	26	26	—	—	—	—	26	—	—	—	—	105
Alloxan resistant	3	3	—	—	—	—	3	—	—	—	—	123

* No islets identified.

† Blood sugar average 280 mg per 100 cc.

‡ " " " " 177 " " " "

as indicated by persistent polyuria, glycosuria, polydipsia, polyphagia, loss of weight and hyperglycemia. Three animals proved to be resistant to the diabetogenic action of alloxan. The latter were killed and examined at intervals of 4, 6 and 11 months respectively after the administration of alloxan.

In Table I are shown the results of the experiments with particular reference to the incidence of hydropic degeneration of the islet and ductular epithelium and the duration of the experiments in which such changes occurred, the duration being calculated from the day of injection of alloxan. The degree and extent of hydropic change was graded histologically on a scale of 0 to 4.

In histological sections of the pancreas, the islets of Langerhans showed varying degrees of reduction in size from animal to animal with from very slight decrease in number to almost total absence of identifiable islets. The hydropic islet lesion consisted of varying degrees of vacuolation of the cytoplasm of the affected cells. The nucleus was centrally located and appeared normal but the cytoplasm in severely affected cells was almost totally replaced and the cell membrane distended by a single vacuole in which no content could be fixed or stained by routine histological methods (Fig. 1C).

The other islet cells that were not affected were readily stained by routine methods and were shown to be almost exclusively alpha cells by the Gomori granule stain. These cells in many islets appeared to be considerably more numerous than could be accounted for by mere reduction in size of the islets from destruction of beta cells and consequent condensation of surviving alpha cells. Although this appearance suggested proliferation of alpha cells, mitotic figures were not found in any of the cells of the islets of Langerhans.

The affected intralobular ductules were only those that are normally lined by cuboidal epithelium. They were found to arise from normal ducts of larger size lined by columnar epithelium and in their finer ramifications to approach, and on occasion even to enter, hydropic islets (Fig. 1A). The earliest noticeable change was a swelling of the cuboidal epithelium that rendered the minute ductules more conspicuous than usual. This was followed by dissolution of the cytoplasm of varying degree up to complete disappearance of the cytoplasm, the place of which was taken by a large clear space bounded by the distended cell membrane (Fig. 1A and B). The centrally placed nucleus appeared slightly smaller and more deeply staining than

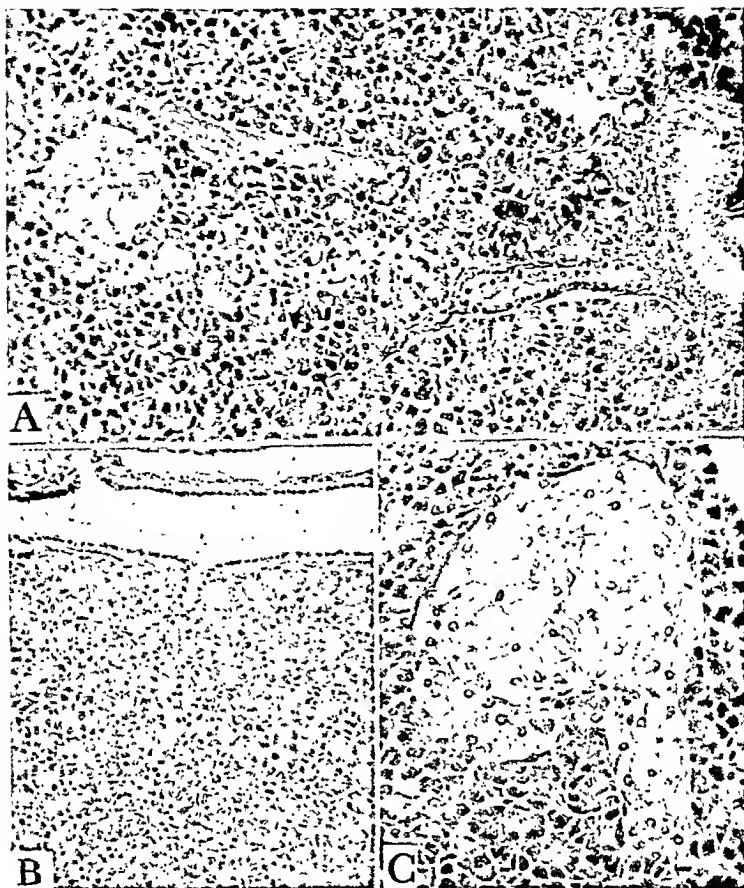


FIG. 1.

Photomicrographs of Sections of Pancreas Stained with Haematoxylin and Eosin.

A. At the right is a duct lined by columnar epithelium of normal appearance. A smaller duct arising from it is lined by cuboidal epithelium, the cells of which show a variable degree of hydropic change. From this latter duct an intralobular ductule exhibiting extreme hydropic degeneration comes into continuity with an islet composed of clear distended hydropic cells and a group of normally stained alpha cells. $\times 165$.

B. A branching intralobular ductule presenting extreme hydropic degeneration of its lining epithelium is shown arising directly from a major duct, above, of which the columnar epithelium is normal in appearance. Note the compact hyperchromatic nuclei of the hydropic cells. $\times 135$.

C. This unusually large islet of Langerhans illustrates the typical appearance of the hydropic changes observed in islet cells. The islet is exceptional in that it contains in the plane of section only two identifiable alpha cells which lie together toward its lower margin. $\times 200$.

normal. The content of the vacuoles, as in the case of the hydropic islet cells, could not be fixed or stained and in both instances special staining to demonstrate glycogen, fat or mucin failed to reveal the presence of any of these substances. The lumina of many affected ductules were dilated by a

small content of acidophilic, homogeneous material. It was found to be impossible in some cases to determine morphologically the islet or ductule origin of many groups of hydropic cells, especially where there was extreme hydropic degeneration in the absence of recognizable alpha cells. It is to

be noted, however, that, with one exception, islet and ductule lesions were found to occur concomitantly. This exception was accounted for by the absence of positively identifiable islets in the sections of pancreas from this one animal.

In addition, a similar hydropic change was observed in isolated single cells, and small groups of cells scattered throughout the acinar tissue. It was impossible to determine morphologically the origin of these cells. However, it was apparent that they were not hydropic acinar cells.

Discussion. Although our experiments were not designed to permit a detailed analysis of the etiological factors concerned in the production of hydropic degeneration of islet and ductule cells of the pancreas, nevertheless, it is apparent that both time and a diabetic state were essential. Table I shows that hydropic degeneration was not found in any of the animals that were diabetic for less than one month. The earliest occurrence of the lesion was found at 45 days, and it was invariably present after 90 days providing that the average fasting blood sugar level during this period had been 303 mg per 100 cc, or higher. One diabetic rabbit with an average blood sugar level of 256 mg showed moderate hydropic degeneration of islet and ductule cells after 12 months. A statistical comparison of the mean of the average blood sugar content of the animals showing hydropic changes of Grades 1 and 2 with that of animals showing Grades 3 and 4 indicated that there was no significant difference between the blood sugar levels of the 2 groups. The degree of hydropic degeneration, therefore, was found to be independent of the degree of hyperglycemia. The absence of hydropic pancreatic lesions in the 3 animals that were found to be resistant to the diabetogenic action of alloxan and in the 2 animals that exhibited relatively mild diabetes, indicated that alloxan *per se* was not the etiological factor concerned.

In the dog and cat, hydropic degeneration of the previously normal beta cells of the islets of Langerhans, whether produced by partial pancreatectomy^{1,2} or by the administration of anterior pituitary extracts,^{3-5,7} is

followed by disintegration of the hydropic cells and a corresponding reduction in size of the islets. This stage of atrophy of islets is reached after 4 to 6 weeks of severe diabetes in the dog,³⁻⁵ but only after 3 to 4 months in the cat.⁷ In contrast to these relatively short periods, it is interesting to note that in alloxan diabetes in the rabbit the hydropic degeneration of the pancreatic islets persists without histological evidence of any disintegration of the affected cells in association with more or less severe diabetes lasting for periods up to one year. However, in this case it is not by any means certain that the hydropic cells are altered beta cells and, indeed, this would appear to be rather doubtful since most, if not all, of the beta cells of the islets were presumably destroyed by the initial administration of a rather large diabetogenic dose of alloxan. In the islets of Langerhans of various species studied histologically 5 days or more after the administration of alloxan several investigators have mentioned the presence of a few indifferent cells with clear, non-granular cytoplasm in addition to the surviving alpha cells.^{6,8,12-14} If in our experiments the numerous vacuolated islet cells arose from the proliferation and subsequent hydropic degeneration of such indifferent cells or of undifferentiated ductular epithelium, these cells might be expected to be no more susceptible to ultimate destruction than the hydropic epithelium of the ductules themselves.

Nevertheless, preliminary experiments have demonstrated by repeated biopsy of the pancreas that the hydropic condition of both islet and ductular epithelium is reversible by adequate treatment of the diabetic state with insulin. Many of the reversed islet cells in the few histological sections of pancreas that we have thus far studied are indifferent cells with clear, nongranular cytoplasm, but others possess a finely granular cytoplasm in-

¹² Gomori, G., and Goldner, M. G., *Proc. Soc. Exp. Biol. and Med.*, 1943, **51**, 287.

¹³ Hard, W. L., and Carr, C. J., *Proc. Soc. Exp. Biol. and Med.*, 1944, **55**, 214.

¹⁴ Duff, G. L., and Starr, H., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 280.

distinguishable from that of the normal beta cells of the islets of normal rabbits. Still other cells present a patchy granularity of the same type and of varying extent, so that all stages of transition from the nongranular cells to cells with a full complement of granular cytoplasm are observed. It is possible that such a transition from nongranular to granular cells of beta type may actually occur under the influence of insulin therapy. This, together with the appearance of proliferation of both of these types of cells, suggests the possibility of improvement or even complete restoration of islet function in relation to carbohydrate metabolism by suitable manipulation of insulin therapy in alloxan diabetes in the rabbit.

Summary. Moderate to extreme hydropic degeneration of the pancreatic ductules and islets was observed in rabbits rendered diabetic by alloxan when the diabetic state had persisted for several months. Such changes have not hitherto been described in alloxan diabetes in the rabbit. The earliest appearance of these alterations was at the end of

45 days after the injection of alloxan and hydropic changes were never absent after 90 days providing that the average fasting blood sugar level during the experiment had been 303 mg per 100 cc, or higher. The hydropic state of the ductules and islets persisted without histological evidence of any further change in association with more or less severe diabetes lasting for periods up to one year. The alpha cells of the islets remained histologically normal but appeared to be increased in number. Preliminary observations demonstrated that the hydropic degeneration of both ductules and islets is reversible by adequate treatment of the diabetic state with insulin. The reversed islet cells appeared to be unduly numerous suggesting proliferation. They were made up in part of indifferent nongranular cells and in part of cells exhibiting varying degrees of granularity of the cytoplasm of beta type. Those with a full complement of granular cytoplasm were indistinguishable in appearance from the beta cells of the islets of Langerhans in the normal rabbit.

15761

Antibacterial Action of N-Alkyl *p*-Aminobenzoic Acid Derivatives.*

G. R. GOETCHIUS AND C. A. LAWRENCE.

From the Sterling Winthrop Research Institute, Rensselaer, N.Y.

Yeomans *et al.*,¹ have recently demonstrated the beneficial therapeutic effect of *p*-aminobenzoic acid in louse-borne typhus fever, and several investigators since then have shown its effectiveness in experimental rickettsial infections.²⁻⁵ In a study on the mode of ac-

tion of sulfonamides, Wyss, Rubin and Strandskov⁶ synthesized several ring-substituted *p*-aminobenzoic acid derivatives and tested them for antibacterial action. They found that the 2-Cl, 3-Cl, 2-NH₂, 3-NH₂, and 3-F derivatives of *p*-aminobenzoic acid displayed varying degrees of bacteriostatic action which could be reversed by the addi-

* The authors wish to express their appreciation to Dr. A. R. Surrey for making available the compounds used in this study. His methods of synthesis appear elsewhere.¹⁰ We are also indebted to Mrs. Beatrice Bass for technical assistance.

¹ Yeomans, A., Snyder, J. C., Murray, E. S., Zarafonitis, C. J. D., and Ecker, R. S., *J. A. M. A.*, 1944, **126**, 349.

² Anigstein, L., and Bader, M. N., *Science*, 1945, **101**, 591.

³ Hamilton, H. L., Plotz, H., and Smadel, J. E., *Proc. Soc. Exp. Biol. and Med.*, 1945, **58**, 255.

⁴ Hamilton, H. L., *Proc. Soc. Exp. Biol. and Med.*, 1945, **59**, 220.

⁵ Murray, E. S., Zarafonitis, C. J. D., and Snyder, J. C., *Proc. Soc. Exp. Biol. and Med.*, 1945, **60**, 80.

⁶ Wyss, O., Rubin, M., and Strandskov, F. B., *Proc. Soc. Exp. Biol. and Med.*, 1943, **52**, 155.

tion of *p*-aminobenzoic acid itself. At pH 7.0 the most active derivative (2-Cl) had a bacteriostatic value equal to sulfapyridine. Hirsch⁷ found *p*-aminobenzamide to be as active as sulfanilamide, and postulated that a new group of substances with chemotherapeutic effects toward bacterial infections might be found among the derivatives of *p*-aminobenzamide.

The present authors investigated the effects upon sulfonamides of a group of *N*-substituted *p*-aminobenzoic acid derivatives and local anesthetics derived from *p*-aminobenzoic acid.^{8,9} During these studies it was noted that several of the compounds, notably the methyl *p*-alkylaminobenzoates, gave evidence of possessing considerable antibacterial activity. It seemed appropriate, therefore, to study more completely the general antibacterial properties of the various alkyl-substituted *p*-aminobenzoic acids.

Methods of Antibacterial Tests. The medium employed throughout these tests was trypticase soy broth (B.B.L.). This medium was found to support excellent growth of all test organisms without the addition of serum or other growth-promoting factors. The organisms employed were as follows: *Pneumococcus* type II, *Streptococcus pyogenes* (C203), *Staphylococcus aureus*, *Haemophilus ducreyi*, *Pasteurella pestis*, *Eberthella typhi*, *Vibrio cholerae*, and *Mycobacterium avium*.

A series of dilutions of the *p*-aminobenzoic acid derivatives ranging from 1:1000 to 1:64,000 was prepared directly in the broth. Owing to the relative insolubility of several of the compounds in water, the initial dilutions were made by dissolving the compounds in a small amount of alcohol followed by the addition of broth. Controls containing an equivalent amount of alcohol had no visible effect upon the growth of the organisms. Tubes containing 5 cc of each dilution were autoclaved at 10 lb for 10 minutes, and

after cooling 0.05 cc of a 1:100 dilution of a 24-hour broth culture of test organism was added to each series. The tubes were incubated at 37°C and examined after 24 hours for visible growth (48 hours in tests employing the pneumococcus, streptococcus and *P. pestis*). Bacteriostasis was recorded for those tubes showing no growth or growth less than half that of the drug-free broth control at the initial observation time. All tubes containing no growth after 72 hours at 37°C were subcultured by transferring 3 4-mm loopfuls to 10 cc of fresh trypticase soy broth. Failure of growth to appear in the subculture tube after 72 hours' incubation was taken as evidence of a bactericidal action by the drug in the original medication tube. Results of these tests are presented in Table I.

Results. It is apparent that the antibacterial activity of *p*-aminobenzoic acid is slightly increased by the substitution of an ethyl or butyl group in the *N*-position. In the series of methyl *p*-alkylaminobenzoates and ethyl *p*-alkylaminobenzoates maximum activity is found in the *N*-propyl-substituted compounds. In this case the *N*-ethyl and *N*-*n*-butyl derivatives are about equal in action which is somewhat less than the *N*-propyl compounds. The series of benzamides display a direct correlation between chain length of the *N*-substituent and degree of antibacterial action. The lowest degree of activity is exhibited by the ethyl derivative with proportionately increasing activity being found in the propyl and *n*-butyl derivatives.

Antagonism by *p*-aminobenzoic acid. Inasmuch as Hirsch⁷ noted that *p*-aminobenzamide was antagonized by the presence of *p*-aminobenzoic acid, and we showed that the *p*-alkylaminobenzamides did not antagonize sulfonamides⁸ it appeared worthwhile to study the effect of *p*-aminobenzoic acid upon the *p*-alkylaminobenzamides, and upon diethylaminoethyl - *p*-*n*-butylaminobenzoate hydrochloride (the *n*-butyl derivative of procaine hydrochloride, U.S.P.). The latter compound had also been found to have none of the sulfonamide antagonizing properties of procaine itself.⁹

Accordingly, diethylaminoethyl-*p*-*n*-butyl-

⁷ Hirsch, J., *Science*, 1942, **96**, 139.

⁸ Goetehius, G. R., and Lawrence, C. A., *J. Bact.*, 1944, **48**, 683.

⁹ Lawrence, C. A., and Goetehius, G. R., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 180.

¹⁰ Surrey, A. R., and Hammer, H. F., *J. Am. Chem. Soc.*, 1944, **66**, 2127.

TABLE I. Antibacterial Action of *p*-Aminobenzoic Acid Derivatives.
 Figures in each left-hand column represent highest dilution \times 1000 exhibiting bacteriostatic action, i.e., 2 = 1:2000, etc. Figures preceded by symbol < denotes highest concentration tested (1:1,000) failed to show evidence of bacteriostasis. Figures followed by asterisk (*) represents highest dilution exhibiting bactericidal activity.

Compound	<i>Pneumococcus</i> type II	<i>Streptococcus</i> <i>pyogenes</i>	<i>Staphylococcus</i> <i>aureus</i>	<i>Haemophilus</i> <i>diteryi</i>	<i>Pasturella</i> <i>pestis</i>	<i>Escherichia</i> <i>typhi</i>	<i>Vibrio</i> <i>cholerae</i>	<i>Mycobacterium</i> <i>avium</i>
<i>p</i> -Aminobenzoic acid	<1	<1	<1	1	1	<1	<1	<1
<i>p</i> -Ethylaminobenzoic acid	2	<1	<1	1	2	<1	<1	<1
<i>p</i> -Butylaminobenzoic acid	2	1	<1	1	4	<1	2	<1
Diethylamino ethyl <i>p</i> -ethylamino benzoate, HCl	4	<1	<1	<1	<1	<1	<1	<1
Diethylamino ethyl <i>p</i> - <i>n</i> -butylamino benzoate, HCl	8	4*	1	2*	2	1	2	1
Methyl <i>p</i> -ethylamino benzoate	2	1	<1	2	2	<1	<1	<1
Methyl <i>p</i> -propylamino benzoate	8	1*	<1	1	8	<1	1*	<1
Methyl <i>p</i> - <i>n</i> -butylamino benzoate	2	1*	<1	2	2	<1	1*	<1
Ethyl <i>p</i> -ethylamino benzoate	16	1*	<1	4	8	<1	1*	<1
Ethyl <i>p</i> -propylamino benzoate	32	1*	<1	4	16	1	2	<1
Ethyl <i>p</i> - <i>n</i> -butylamino benzoate	16	4	<1	32	4	<1	1	<1
Ethylamino benzamide	2	<1	<1	1	1	<1	<1	<1
<i>p</i> -Ethylamino benzamide	4	2*	<1	2	1	1	1*	<1
<i>p</i> -propylamino benzamide	8	4	<1	4	1	1	2	<1
<i>p</i> - <i>n</i> -Butylamino benzamide							1*	<1

aminobenzoate hydrochloride and *p*-*n*-butylaminobenzamide were dissolved in 1% concentration directly into trypticase soy broth containing 10^{-3} , 2×10^{-4} , 10^{-4} , and 2×10^{-5} dilutions of *p*-aminobenzoic acid. The initial concentrations of the 2 test compounds were then diluted serially up to and including dilutions of 1:12,800. The compounds were also diluted in a similar manner in trypticase soy broth containing no *p*-aminobenzoic acid for control titrations. The tubes were autoclaved at 10 pounds for 10 minutes, and after cooling were inoculated with a 4-mm loopful of a 24-hour broth culture of *Streptococcus pyogenes* (C203). The inoculated tubes were incubated as before and the bacteriostatic and bactericidal end-points recorded.

Results. The data obtained in this study revealed that *p*-aminobenzoic acid does not antagonize the antistreptococcal activity of either diethylaminoethyl-*p*-*n*-butylaminobenzoate hydrochloride or *p*-*n*-butylaminobenzamide. It would appear from these findings, therefore, that N-alkylation of these *p*-aminobenzoic acid derivatives not only nullifies their antagonistic action upon sulfonamides, but also gives their own antibacterial activity an "immunity" to antagonism by *p*-aminobenzoic acid.

Summary. 1. The bacteriostatic and bactericidal activity of a series of N-substituted *p*-aminobenzoic acid derivatives have been determined.

2. Among the acids the order of decreasing activity is as follows: *p*-butylaminobenzoic acid > *p*-ethylaminobenzoic acid > *p*-aminobenzoic acid.

3. Of 2 procaine hydrochloride (U.S.P.) derivatives, the N-butyl derivative displays greater antibacterial action than the N-ethyl.

4. Among the methyl and ethyl esters of *p*-aminobenzoic acid, the N-propyl derivatives display more activity than the N-ethyl or N-butyl compounds. The latter 2 were approximately equal in respect to antibacterial action.

5. The order of decreasing activity among the *p*-alkylaminobenzamides was: butyl > propyl > ethyl.

tion of *p*-aminobenzoic acid itself. At pH 7.0 the most active derivative (2-Cl) had a bacteriostatic value equal to sulfapyridine. Hirsch⁷ found *p*-aminobenzamide to be as active as sulfanilamide, and postulated that a new group of substances with chemotherapeutic effects toward bacterial infections might be found among the derivatives of *p*-aminobenzamide.

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after cooling 0.05 cc of a 1:100 dilution of a 24-hour broth culture of test organism was added to each series. The tubes were incubated at 37°C and examined after 24 hours for visible growth (48 hours in tests employing the pneumococcus, streptococcus and *P. pestis*). Bacteriostasis was recorded for those tubes showing no growth or growth less than half that of the drug-free broth control at the initial observation time. All tubes containing no growth after 72 hours at 37°C were subcultured by transferring 3 4-mm loopfuls to 10 cc of fresh trypticase soy broth. Failure of growth to appear in the subculture tube after 72 hours' incubation was taken as evidence of a bactericidal action by the drug in the original medication tube. Results of these tests are presented in Table I.

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Accordingly, diethylaminoethyl-*p*-*n*-butyl-

⁷ Hirsch, J., *Science*, 1942, **96**, 139.

⁸ Goetchius, G. R., and Lawrence, C. A., *J. Bact.*, 1944, **48**, 683.

⁹ Lawrence, C. A., and Goetchius, G. R., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 180.

¹⁰ Surrey, A. R., and Hammer, H. F., *J. Am. Chem. Soc.*, 1944, **66**, 2127.

TABLE I.
Influence of Pathogenizing Substances on Agglutination of the 0901 Strain by Anti-O Serum
(Titer 1:10,000).

Substance tested	Concentration, %	End of the agglutination titer
Dextran (<i>L. mesenteroides</i>)	0.1-0.25	1:10,000
	0.5-1.0	1: 1,000
	2.0	negative at 1:100
Levan (<i>A. levanicum</i>)	0.1-0.5	1:10,000
	1.0	1: 5,000
	2.0	negative at 1:100
Mucin	1.0	1:10,000
	2.0	1: 2,000
	5.0	negative at 1:100
Pectin	0.1-0.2	1:10,000
	0.5	1: 1,000
	1.0	1: 100
	2.0	negative at 1:100
Glycogen	0.5	1:10,000
	1.0	1: 2,000
	2.0	1: 1,000
Starch	1.0-2.0	1:10,000
	5.0	1: 200
Inulin, gum acacia, cellulose (cotton)	0.1-5.0	1:10,000

ence of any of the inhibiting substances (dextran, levan, mucin, pectin), however, no measurable quantities of antibody N were taken up by the cells. These observations accord with the findings of Keefer and Spink⁴ concerning the effect of mucin on the bacteriolysis of gonococci by whole blood and immune serum.

⁴ Keefer, C. S., and Spink, W. W., *J. Clin. Invest.*, 1938, 17, 23.

Summary. The union of O-antibody by *E. typhosa* is completely inhibited by dextran, levan, and pectin at 2% concentration and by mucin at 5% concentration. Reduction of agglutination titer occurs in the presence of starch (5%) and glycogen (1-2%). Inulin, gum acacia, and acid-degraded cotton cellulose in 5% concentration are without this effect.

15763

Influence of Nitrogen Mustards on the Antibody Response.*

CHARLES L. SPURR. (Introduced by E. S. Guzman-Barron.)

From the Department of Medicine, the University of Chicago, Chicago, Ill.

The absence of infection in the leucopenic phase following nitrogen mustard therapy of lymphomas suggests that some specific pro-

tective mechanism persisted despite the demonstrable widespread damage to the hemopoietic system. The role of lymphocyte in the antibody response^{1,2} suggests the lym-

* This study was supported in part by a grant from the Committee on Growth, American Cancer Society.

¹ Ehrlich, W. E., and Harris, T. N., *J. Exp. Med.*, 1942, 76, 335.

6. Diethylaminoethyl-*p-n*-butylaminoben-zate hydrochloride and *p-n*-butylaminoben-zamide were not antagonized by the presence

of *p*-aminobenzoic acid when tested in trypticase soy broth against *Streptococcus pyo-genes* (C203).

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Action of Certain Carbohydrates on the Reaction of *Eberthella typhosa* with Antibody O.

L. OLITZKI, M. SHELUBSKY, AND E. EFRATI.

From the Department of Hygiene and Bacteriology, The Hebrew University of Jerusalem.

In a previous publication, Olitzki, Shelubsky and Hestrin¹ reported that certain carbohydrates promote the pathogenicity of *E. typhosa*. Since Gram-negative microorganisms are normally eliminated from the peritoneal cavity in the mouse mainly by bacteriolysis a short time after the onset of infection,² the possibility that the carbohydrates, which alter pathogenicity might also interfere with a bacteria-antibody reaction seemed to deserve consideration. The influence of the carbohydrates previously employed in tests of pathogenicity on the agglutination of typhoid strain 0901 by its O-antibody, the presence of which in antisera parallel the bactericidal activity,³ has accordingly been examined. The results of the agglutination tests are shown in Table I.

Table I shows that the carbohydrates used for testing, *viz.* dextran, levan, mucin and pectin, markedly inhibit agglutination. The results were the same whether bacteria were added to the serum-carbohydrate mixture immediately after its preparation or after it had been incubated for 4 hours at 37°C.

The mechanism of the inhibition could be a serological reaction between the test carbohydrates and the typhoid antibody. We have not, however, been able to detect a visible precipitation reaction when the car-

bohydrates were mixed with anti-typhoid serum. In order to ascertain whether the inhibition is produced by interference on the part of the carbohydrates with fixation of antibodies by the bacterium, or by an intervention in the second stage of the agglutination reaction, we measured the effect of the more active of the inhibitors (dextran 2%, levan 2%, pectin 2%, and mucin 5%) on antibody-nitrogen uptake.

The carbohydrate solutions were sharply centrifuged to remove all insoluble particles. Heat-killed and washed bacteria sediment containing 0.419 mg nitrogen was then suspended in 2.0 cc of the solutions. After the selected carbohydrates and bacteria had been in contact for 2 hours at 37°C, the immune serum was added and the mixture allowed to stay for 24 hours in the ice box. The bacteria were then removed and washed and the N-uptake determined by a Micro-Kjeldahl method. In another series of experiments the serum was left in contact with an inhibitor for 2 hours at 37°C. Examination of this mixture failed to reveal any visible precipitation. Bacterial sediment containing 0.260 mg nitrogen was then suspended in the mixture. The suspension was left for 24 hours in the ice box. The bacteria were then removed and examined as in the previous series. Both series of experiments gave similar results. The control test without the addition of inhibitor showed that the bacteria are normally able to remove in 24 hours 0.033 mg N from 0.1 ml serum and 0.061 mg N from 0.2 ml serum. In the pres-

¹ Olitzki, L., Shelubsky, M., and Hestrin, S., in press.

² Olitzki, L., and Koch, P. K., *J. Immunol.*, 1945, **50**, 229.

³ Felix, A., and Olitzki, L., *J. Immunol.*, 1926, **11**, 31.

per kg weight (total dose 1 mg) was given every 4 days for 4 weeks. The antibody titre rose to 1-160 as compared to a control titre of 1-1280.

Comment. The studies of Ehrich¹ have shown that the lymphocytes from lymph of a regional node draining an extremity into which antigen has been injected contain an increased titre of antibody on the fourth to sixth day. Dougherty and White^{2,3} have shown that corticosterone will produce a lymphopenia and that the decrease in these cells, with their dissolution is associated with a rise in the antibody titre. Since the nitrogen mustards produce a toxic lymphopenia which is apparent in the peripheral blood⁴ and lymph node⁵ within 4 days, it might be expected that this toxic dissolution would result in the increase of antibody. With this in view, the nitrogen mustard was given after the normal antibody curve had fallen but no rise in titre similar to the anamnestic reaction was seen. No summation of titre occurred when nitrogen mustard

was given at the peak of the antibody response.

These findings do not contest the role of the lymphocyte in antibody formation but suggest that the toxicity of nitrogen mustard interferes with the antibody forming mechanism of the lymphocyte. This hypothesis is supported by the suppression of the antibody formation in animals receiving antigen following pretreatment and concurrent treatment with nitrogen mustard. The dosage used in these experiments are 10-fold or more than that given in human therapy and an investigation of the antibody response in the human is now in progress. It is of interest to mention that in the human given a course of 25 mg nitrogen mustard, the leucopenic phase coincides with a period of active regeneration of the hemopoietic system.⁶

Summary. 1. The lymphocytotoxic effect of the nitrogen mustards will not produce an anamnestic reaction or summation of the antibody titre in the rabbit.

2. Pretreatment and concurrent administration of nitrogen mustard suppress the antibody response to typhoid antigen.

³ Dougherty, T. F., White, A., and Chase, S. H., *Proc. Soc. Exp. Biol. and Med.*, 1944, 56, 28.

⁴ Jacobson, L. O., Spurr, C. L., Barron, E. S. G., Smith, T. R., Lushbaugh, C., and Dick, G. F., *J. A. M. A.*, 1946, 132, 263.

⁵ To be published.

⁶ Spurr, C. L., Jacobson, L. O., Smith, T. R., and Barron, E. S. G., A.A.A.S. Gibson Island Conf. on Cancer, *Chemotherapy of Tumors*, in press; *Cancer Research*, 1947, 7, 51.

15764

Chemotherapeutic Action of Streptomycin and of Streptomycin with a Sulfone or Sulfadiazine on Tuberculosis.

M. I. SMITH, W. T. McCLOSKEY, E. L. JACKSON, AND H. BAUER.

From the Division of Physiology, National Institute of Health, Bethesda, Md.

Previously published experiments¹ have shown that streptomycin and promin used together in the treatment of experimental tuberculosis in guinea pigs produced a chemotherapeutic effect greater than the sum of effects from the individual components.

Equally good results were later reported from the combined use of streptomycin and another sulfone, sodium salt of 4-amino,4'-galacturonylamindiphenylsulfone.² The supply of streptomycin, however, was then so limited that no experiments could be made

¹ Smith, M. I., and McCloskey, Wm. T., *Pub. Health Rep.*, 1945, 60, 1129.

² Smith, M. I., McCloskey, W. T., and Jackson, E. L., *Am. Rev. Tub.*, in press.

phocytotoxic nitrogen mustard compounds may influence the antibody reaction.

Method. To elucidate this relationship, the response to typhoid vaccine 1 cc intramuscularly, in a single dose and in 3 weekly doses was followed at 3-7-day intervals by agglutination titration with dilutions of 1 to 10 to 1 to 5120. A control group of 3 rabbits was given typhoid vaccine 1 cc intramuscularly and the agglutination titre followed through its peak response of 1 to 1280 on the 21st day until the titre had fallen. On the 80th to 95th day, nitrogen mustard (methyl bis β chlorethyl amine) 1 mg per kg (total dose 2 mg) was injected intravenously every 5 days for 4 doses. No increase in the antibody titre similar to the anamnestic reaction was observed. A second control group of 3 rabbits received injections of 1 cc typhoid vaccine intramuscularly for 3 weeks with a resulting increase in antibody titre to 1-5120 in the third week. Nitrogen mustard 1 mg every 4 days was given for 3 doses. There was no summation of titre but a decline to 1-1280 over the following week occurred.

A group of 3 rabbits was given nitrogen mustard at a dose of 0.5 mg per kg weight (1 mg total dose) every 4 days for 9 weeks. On the 25th day, when the lymphocyte count had fallen to 30% of the pretreatment level, typhoid vaccine 1 cc was given intramuscularly. The antibody titre increased only 1-80 3 weeks later, while the controls reached a titre of 1-1280. Another group of 3 rabbits received a similar dose of nitrogen mustard for 7 weeks. On the 18th day, 3 weekly doses of 1 cc typhoid vaccine were initiated. The treated group showed an antibody titre of 1-320 in the third week as compared to a control level of 1-5120.

The antibody response following simultaneous injection of typhoid vaccine and nitrogen mustard was investigated to determine to what extent this cytotoxic material might interfere with the antibody response. Typhoid vaccine 1 cc intramuscularly was given on the first day and nitrogen mustard 0.5 mg

TABLE I
Typhoid Agglutination Titers Following Intravenous Injection of Nitrogen Mustard.

Group	No. rabbits	Weeks												
		0	1	2	3	4	5	6	7	11	12	13		
1	Control anamnestic	*												
2	Control summation	0	1/320	1/640	1/1280	1/640	1/320	1/80	1/10	1/10	1/10	1/10	1/10	0
3	Preatigen and concurrent	0	1/80	1/640	1/5120	1/1280	Died							0
4	Preatigen and concurrent	0	0	0	0*	0	1/40	1/80	1/40	1/40	1/10	1/10		10
5	Simultaneous antigen and nitrogen mustard	0*	1/20	1/20	1/160*	1/320*	1/80*	1/40						
			1/20	1/20	1/160	1/80	1/160	1/80	1/160	1/80	1/80	1/80		

* Typhoid vaccine 1 cc, 500 million bacteria intramuscularly.

— Methyl-bis- β chlorethyl-amine hydrochloride 2 mg intravenously every 5 days, 4 doses.

— Nitrogen mustard 1 mg every 4 days.

TABLE I.

The Tuberculin Reaction (PPD) and the Extent of Tuberculous Involvement (Tuberculosis Index, T.B.I.) at Autopsy in the Individual Animals in the Several Groups Treated with Streptomycin Alone or in Combination with Derivatives of Diaminodiphenylsulfone (DDS) or Sulfadiazine. All Animals Inoculated with 0.4 mg H37Rv Intraperitoneally.

No.	G		A		B		C		D		E		F		L	
	Controls T.B.I.	Streptomycin PPD T.B.I.	Streptomycin + n-propyl DDS PPD T.B.I.	Streptomycin + succinimido DDS PPD T.B.I.	Streptomycin + n-propyl DDS PPD T.B.I.	Streptomycin + sulfadiazine PPD T.B.I.	Streptomycin + sulfadiazine PPD T.B.I.	Streptomycin + n-propyl DDS T.B.I.	Succinimido DDS T.B.I.	Streptomycin, 23 days after infection T.B.I.						
1	13	+	0	+	*	+	+	+	+	+	+	+	+	+	0	0
2	6	+	0	+	0	+	+	+	+	+	+	+	+	+	2	2
3	10	+	+	+	0	+	+	+	+	+	+	+	+	+	6	6
4	20	+	+	+	0	+	+	+	+	+	+	+	+	+	±	±
5	5	+	+	+	0	+	+	+	+	+	+	+	+	+	2	2
6	19	0	0	+	0	+	+	+	+	+	+	+	+	+	±	±
7	20	0	0	+	0	+	+	+	+	+	+	+	+	+	5	5
8	12	+	2	+	0	+	+	+	+	+	+	+	+	+	13	13
9	20	+	2	+	1	+	+	+	+	+	+	+	+	+	14	14
10	15	0	0	+	±	+	+	+	+	+	+	+	+	+	1	1
11	9	+	1	+	0	+	+	+	+	+	+	+	+	+	10	10
12	18	+	1	+	0	+	+	+	+	+	+	+	+	+	11	11
13	20	+	2	+	±	+	+	+	+	+	+	+	+	+	15	15
14	11	0	1	+	0	+	+	+	+	+	+	+	+	+	8	8
15	20	+	1	+	±	+	+	+	+	+	+	+	+	+	2	2
16	11	+	0	+	3	+	+	+	+	+	+	+	+	+	5	5
17	16	+	2	+	2	+	+	+	+	+	+	+	+	+	8	8
18	20	+	±	+	1	+	+	+	+	+	+	+	+	+	10	10
19	10	+	2	+	0	+	+	+	+	+	+	+	+	+	13	13
20	13	+	1	+	1	+	+	+	+	+	+	+	+	+	3	3
Avg	14.4	—	1.0	—	0.5	—	0.8	—	1.0	—	3.9	—	7.9	—	2.4	2.4

* Died accidentally at an early date.

with streptomycin alone to appraise fully the value of the combined treatment.

Data have been accumulated on the relative chemotherapeutic value of several sulfone derivatives with the object of finding a compound less toxic, and if possible more effective, than promin or the parent substance 4,4'-diaminodiphenylsulfone (DDS). Studies on this phase of the problem have indicated that the mono *n*-propyl derivative of DDS was much less toxic than promin and at least as effective as the latter.² Another derivative of DDS, the mono-succinimide, synthesized in this laboratory by one of us (H.B.), was also found to have a low degree of toxicity and appeared worthy of therapeutic trial with streptomycin. A trial experiment with streptomycin and a sulfonamide also appeared desirable, especially since sulfadiazine had previously given some indication of chemotherapeutic activity in experimental tuberculosis, even if only of a very low order, compared with the sulfones.³

Accordingly a series of experiments were set up to determine (a) the chemotherapeutic effectiveness of streptomycin alone at a dose level of 40 mg per kg per day, a dose 2 to 4 times as great as had been used hitherto, (b) the effect of streptomycin at half the above dose in combination with one of the 2 sulfones mentioned earlier or with sulfadiazine.

Experimental. A series of male guinea pigs weighing 300 to 350 g, usually about 325 g, were inoculated intraperitoneally with 1 cc of a homogeneous bacillary suspension in saline containing 0.4 mg moist weight of tubercle bacilli, human strain, H37Rv.* The animals were divided into 6 groups of 20 each and treated as follows:

Group A. Streptomycin, 20 mg[†] per kg intramuscularly twice daily, 9 a. m. and 4 p. m.

Group B. Streptomycin, 10 mg per kg intramuscularly twice daily, and *n*-propyl de-

rivative of diaminodiphenylsulfone (*n*-propyl),[‡] 500 mg per kg per day, orally.

Group C. Streptomycin as in Group B and 250 mg per kg per day of the succinimido derivative of diaminodiphenylsulfone (succinimido)[§] orally.

Group D. Streptomycin as in Group B and 500 mg per kg per day of sulfadiazine orally.

Group E. *n*-Propyl as in Group B but no streptomycin.

Group F. Succinimido as in Group C, but no streptomycin.

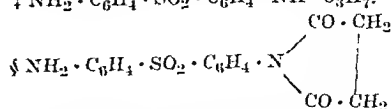
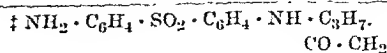
Group G. Untreated controls.

The sulfones and sulfadiazine were administered by stomach tube in 10% aqueous suspension with 5% gum acacia.

Treatment was begun the day after inoculation and continued for 11 weeks, 5 days a week, with a double dose on the fifth day. Three weeks after treatment was discontinued the survivors were tuberculin tested, using 0.01 mg PPD in 0.1 cc saline intracutaneously. Readings were made 24 and 48 hours later. Eighteen to 20 weeks after infection all the survivors were killed with chloroform, autopsied, and the extent of tuberculous involvement recorded as previously described.⁵

In addition to the 6 foregoing groups a small group (L) of 7 guinea pigs was set aside for treatment, beginning 23 days after infection with 10 mg per kg streptomycin twice daily. Treatment in this group was continued for 11 weeks as in the preceding groups. These animals were killed and autopsy findings recorded as in the others.

Results. Table I gives in detail the extent of tuberculous involvement (tuberculosis index, T.B.I.) in the individual animals in each group at autopsy and also the average for each group. The ratings are all based on a possible maximum of 20. The sign \pm



³ Smith, M. I., Emmart, E. W., and Westfall, B. B., *J. Pharm. and Exp. Therap.*, 1942, **74**, 163.

* Courtesy Mr. W. Steenken, Jr., acting for the Committee on Standard Cultures of the Medical Research Committee of the National Tuberculosis Association.⁴

[†] 1 mg = 1000 units.

⁴ A Depot for Standard Cultures of Tubercle Bacilli, *Am. Rev. Tub.*, 1946, **53**, 511.

⁵ Smith, M. I., Emmart, E. W., and Stohlman, E. F., *Am. Rev. Tub.*, 1943, **48**, 32.

TABLE I.

The Tuberculin Reaction (PPD) and the Extent of Tuberculous Involvement (Tuberculosis Index, T.B.I.) at Autopsy in the Individual Animals in the Several Groups Treated with Streptomycin Alone or in Combination with Derivatives of Diaminodiphenylsulfone (DDS) or Sulfadiazine. All Animals Inoculated with 0.4 mg H37Rv Intraperitoneally.

No.	G		A		B		C		D		E		F		L	
	Controls T.B.I.	Streptomycin PPD	T.B.I.	Streptomycin + n-propyl DDS PPD	T.B.I.	Streptomycin + succinimido DDS PPD	T.B.I.	Streptomycin + sulfadiazine PPD	T.B.I.	n-Propyl DDS T.B.I.	Succinimido DDS T.B.I.	Streptomycin, 23 days after infection T.B.I.				
1	13	+	0	*	+	+	+	+	+	2	9	0				
2	6	+	0	+	+	+	+	+	2	2	3	2				
3	10	+	+	+	+	+	+	+	6	6	4	2				
4	20	+	+	+	+	+	+	+	3	3	11	6				
5	5	+	+	+	+	+	+	+	1	1	6	6				
6	19	0	1	+	+	+	+	+	3	3	14	2				
7	20	0	0	0	+	+	+	+	3	3	2	2				
8	12	+	2	0	+	+	+	+	1	1	13	1				
9	20	+	2	+	+	+	+	+	3	3	14	1				
10	15	0	0	+	+	+	+	+	2	2	10	2				
11	9	+	1	+	+	+	+	+	6	6	11	2				
12	18	+	1	0	+	+	+	+	4	4	11	2				
13	20	+	2	+	+	+	+	+	2	2	15	8				
14	11	0	1	+	+	+	+	+	9	9	8	2				
15	20	+	1	*	+	+	+	+	5	5	2	2				
16	11	+	0	3	+	+	+	+	6	6	8	2				
17	16	+	2	2	+	+	+	+	12	12	10	1				
18	20	+	2	1	+	+	+	+	1	1	13	2				
19	10	+	2	+	+	+	+	+	2	2	3	6				
20	13	+	1	+	+	+	+	+	6	6	3	—				
Avg	14.4	—	1.0	0.5	—	—	—	—	1.0	3.9	7.9	2.4				

* Died accidentally at an early date.

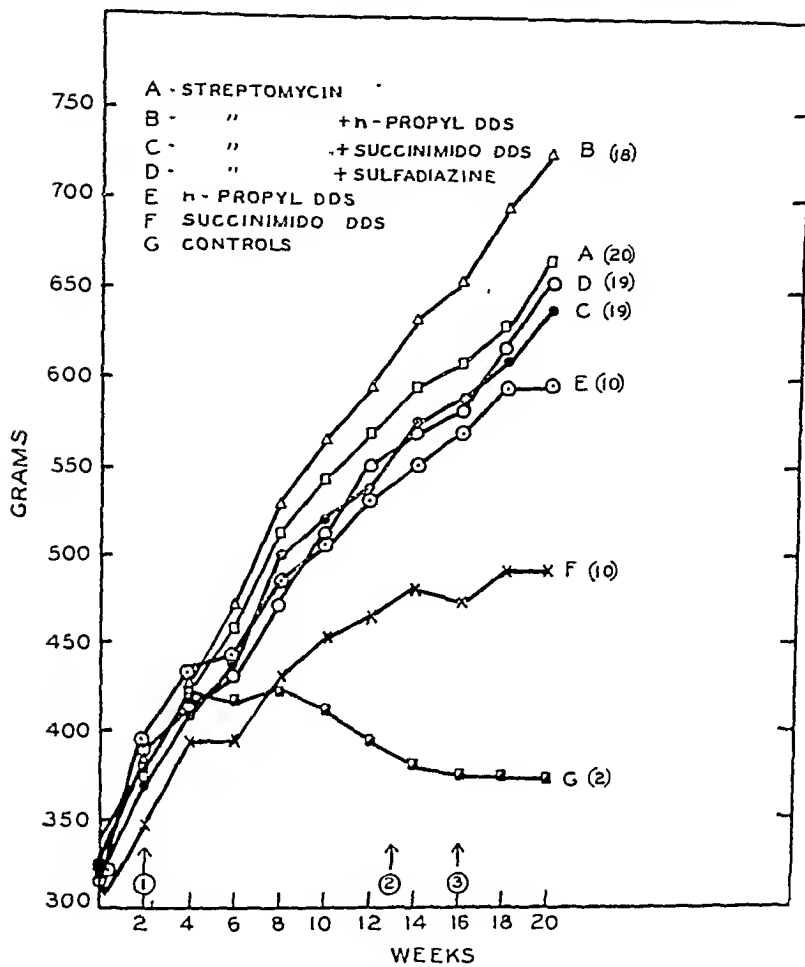


FIG. 1.

Average weight curves of groups of tuberculous guinea pigs treated with streptomycin (A), streptomycin and a derivative of diaminodiphenylsulfone (B and C), streptomycin and sulfadiazine (D), sulfone derivatives alone (E and F). G represents a group of untreated controls. Figures in parentheses indicate number of animals surviving at termination of experiment. First arrow shows time of infection and when treatment was begun; second arrow indicates end of treatment; at third arrow the survivors were tuberculin tested.

For details as to dosage and other pertinent data see Table II.

designates doubtful lesions and has been arbitrarily assigned a numerical value of 0.5. The results of the tuberculin tests for Groups A, B, C, and D are given in the PPD columns of the respective groups.

Group G Controls. Eighteen animals in this group (90%) died with extensive generalized tuberculosis in from 44 to 97 days. The remaining 2 animals were killed at the termination of the experiment, 126 days after infection, and these, also, had advanced tuberculosis of the viscera. The tuberculosis index in the individual animals varied from

5 to 20 (out of a possible maximum of 20) with an average of 14.4.

Group A. All the animals treated with 20 mg per kg streptomycin twice daily survived the experimental period, and made good gains in weight, as shown in curve A, Fig. 1. Though 14 animals in this group gave positive tuberculin reactions, 8 (40%) showed none or only doubtful lesions at necropsy. The remainder had a tuberculosis index of 1 to 2, with an average of 1 for the entire group.

Group B. Two of the animals treated with

streptomycin and the *n*-propyl compound died in 27 and 32 days respectively. At autopsy there were emphysema and congestion of the lungs in one and pulmonary edema in the other. The animals had shown no signs of illness up to the last drug feeding by stomach tube, and death in these 2 animals was regarded as accidental. The remaining 18 animals in this group gained weight normally, survived the full experimental period, and while 10 of them gave positive tuberculin reactions, 13 (72%) showed no gross evidence of tuberculosis at autopsy. The animals in this group showed the highest average gain in weight (curve B, Fig. 1). The average tuberculosis index for the entire group was 0.5.

Groups C and D. The animals treated with streptomycin in combination with the succinimido compound and sulfadiazine respectively compared favorably with the animals in Group A receiving streptomycin alone at twice the dose level. One animal in each of these groups died, at 71 and 104 days respectively, and while pulmonary emphysema was present in both neither showed much evidence of tuberculosis. The average tuberculosis index for Group C was 0.8, and 11 animals (55%) showed no gross evidence of tuberculosis. Ten animals in this group gave positive tuberculin reactions. The average tuberculosis index for Group D was 1.0, with 10 animals (50%) showing no macroscopic lesions, and 9 reacting to tuberculin. The weight curves of the animals of these 2 groups were nearly identical with that of Group A.

Groups E and F. Treatment with the *n*-propyl and succinimido derivatives respectively resulted in the mortality of 50% in each group. However, all of the survivors in Group E were in very good condition, while 4 of the survivors in Group F at the termination of the experiment were losing weight. The average weight curve for Group E was decidedly better than that of Group F. At autopsy all the animals in both groups had definite macroscopic tuberculosis. However, the average tuberculosis index for Group E was 3.9 with a variation of 1 to 12, while

TABLE II.
Summary of All Tests in the Several Groups.

Group and drug, mg/kg/day	B					
	C	A	2 × 10 + <i>n</i> -Propyl DDS	Streptomycin 2 × 10 + succinimido DDS	C	D
	Controls	Streptomycin 2 × 20	Streptomycin 2 × 10 + <i>n</i> -Propyl DDS	Streptomycin 2 × 10 + succinimido DDS	E	F
Mortality % at termination of exp. 130-140 days after infection	90	0	0	5	50	50
Avg T.B. index computed on basis of 100 for controls	100	6.9	3.5	5.5	26.9	54.5
Percent with no or doubtful lesions (T.B. rating of 0 or ±)	0	40	72	55	0	0
Chemotherapeutic effectiveness (ratio of T.B. index of controls and treated groups)	53	14.4 342	28.6 402	18.2 332	3.7 278	1.8 207
Avg wt gains, g	1.0-12.0	0.7-1.8	0.7-1.6	0.7-1.4	0.9-7.0	0.7-9.9
Wt of spleen, g	5.4	1.0	1.2	0.9	2.8	2.5
Range						
Avg						

TABLE III.
Blood Levels. Average of 3 to 4 Animals.

Drug	Daily dose, g/kg	Mg %, hr after administration		
		3	5	19-24
<i>n</i> -Propyl DDS	0.25	1.5	1.5	0.8
	0.50	2.0	2.1	1.2
	2.00	3.4	3.6	3.8
Succinimido DDS	0.25	1.8	0.7	0.5
	2.00	1.6	1.5	1.6

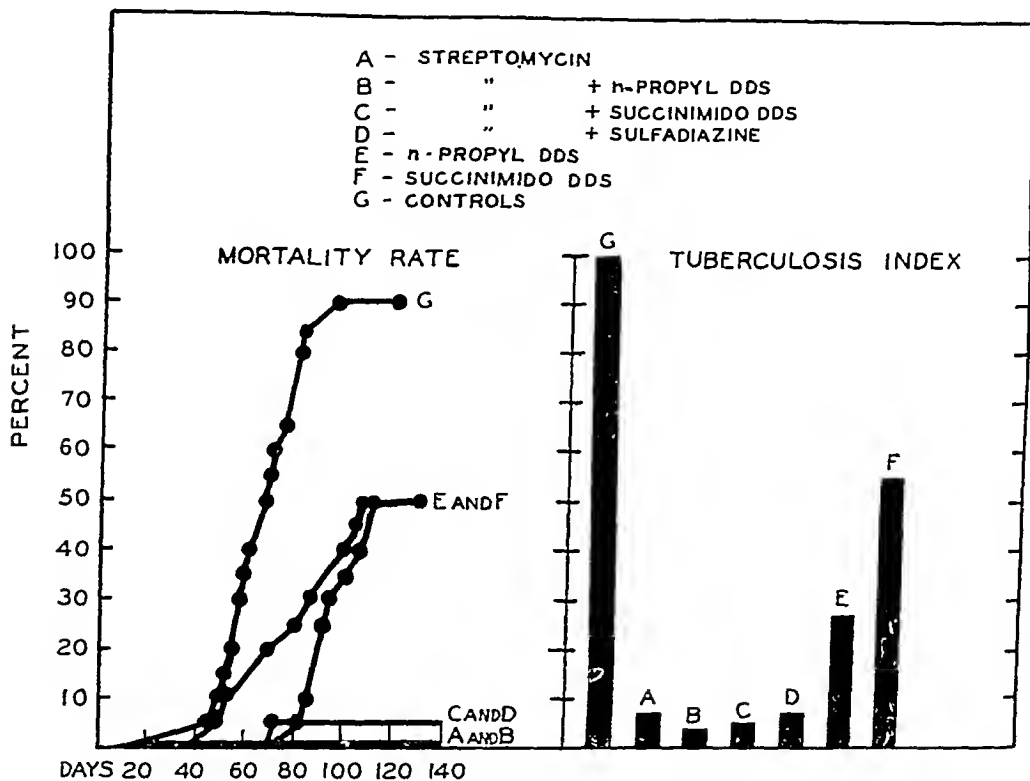


Fig. 2.

Mortality rate and average extent of tuberculous involvement (tuberculosis index) in groups of tuberculous guinea pigs treated as indicated in legend of Fig. 1.

in Group F the average index was 7.9 with a variation of 1 to 15. This evidence appears to indicate that while neither drug eradicated the disease the *n*-propyl derivative had the much greater suppressive effect of the 2.

Group L. The animals in this group in which treatment was begun 23 days after infection all survived the experimental period, with marked improvement in their weight curve after treatment was instituted, and necropsy at the termination of the experi-

ment showed an average tuberculosis index of 2.4 with a variation of 0 to 6. Two of the 7 animals showed no gross evidence of the disease.

Tissues from each of the 7 animals in this group, together with tissues from 5 animals of the control group, were submitted to Dr. R. D. Lillie of the Laboratory of Pathology for microscopic examination. These included liver, spleen, lungs, kidneys, testes, omentum, and diaphragm. His re-

port is summarized as follows:

Of the 5 controls one showed chronic progressive tuberculosis, 2 had subacute progressive tuberculosis, and 2 showed active uninhibited tuberculosis. Two of the treated animals (L 1 and L 5) had no tuberculosis, the latter showing subacute bronchopneumonia. The others presented the following:

L 2. Solitary tubercle of spleen, relatively inactive.

L 3. Old lymphadenoid tuberculosis, relatively inactive.

L 4. Inactive old tuberculosis with calcification and fibrosis in the lungs and lymph nodes. Chronic bronchopneumonia and bronchiectasis.

L 6. Old lymphadenoid and peritesticular tuberculosis with some calcification and fibrosis and chronic interstitial pneumonia.

L 7. Minimal chronic tuberculosis of the lungs, spleen and mesenteric lymphnodes with bronchopneumonia and bronchiectasis.

These findings agree well with the data given in Table I, and recorded independently on the basis of gross observation.

In Table II are summarized the results of the entire experiment. Comparison of the relative values of the different forms of treatment, based on the most important criteria, namely mortality per cent, average tuberculosis index, and incidence of freedom from disease as judged by macroscopic examination, all point to the superiority of combined treatment with streptomycin and the *n*-propyl derivative of diaminodiphenylsulfone. The average gains in weight of the animals in the several groups as shown in Table II and Fig. 1 also point to the same conclusion.

Of the 2 sulfones presented in this study the data in Tables I and II and in Fig. 1 and 2 indicate that the *n*-propyl derivative has the greater suppressing effect on the disease. Both compounds have a low degree of toxicity and are characterized by poor absorbability, as shown in Table III. It is to be noted that the succinimido compound was used in the therapeutic tests at half the dose level of the *n*-propyl derivative, but it is doubtful whether it would have been more effective at the higher dose level, since in normal animals little more of this drug was

absorbed when administered in daily doses of 2.0 g per kg than when given in doses of 0.25 g per kg.

Discussion. Viewing the results of the present study against the background of those previously reported it is becoming increasingly evident that streptomycin is by far the most effective chemotherapeutic agent in experimental tuberculosis. Working under fairly well standardized conditions, with a rather heavy infection giving a mortality of from 65 to 95% of the controls in 90 to 100 days, treatment with streptomycin at a dose level of 10 to 15 mg per kg per day gave a chemotherapeutic effectiveness of 5.2, no mortality within the experimental period, and absence of macroscopic tuberculosis in 15% of the animals.¹ When the daily dose of streptomycin was increased to 40 mg per day in the present series under very similar conditions a chemotherapeutic effectiveness of 14.4 was obtained, no mortality, and 40% of the animals appeared to be free from lesions. This latter dose of streptomycin is approximately 1/10 of the LD₅₀.¹ It is evident therefore that the response is roughly proportional to dosage, and it is not impossible that further improvement may be had with increasing dosage. It is to be remembered however, that in all cases treatment was begun the day after infection and continued for 60 to 80 days. The picture is not as bright however, when treatment is delayed, as it was done in the present study, Group L, where treatment with streptomycin was not begun until 23 days after infection in which case 5 of the 7 animals had slight to moderate degrees of tuberculosis and a chemotherapeutic effectiveness for the group of only 6.0 (14.4/2.4) was obtained.

Summarizing the present and previous results on the action of streptomycin and the sulfones used individually or in combination it appears that there is good evidence of potentiation every time combined therapy has been tried. Obviously the better the sulfone the more impressive the effect. For convenience the essential data of the present study and of the 2 previous publications^{1,2} have been put together in Table IV. Expressing the value of a given treatment

TABLE IV.
Summary of Essential Data on the Effects of Streptomycin Used Alone or in Combination with a Derivative of Diaminodiphenylsulfone in Experimental Tuberculosis.

Streptomycin mg/kg/day	Sulfone derivative	Chemotherapeutic effectiveness	Mortality %	Free from lesions, %	Mortality, % of controls	Duration of exper., days	Reference
10-15	0	5.2	0	15	65	110	1
0	Promin	2.4	15	5	65	110	1
10-15	"	20.0	0	65	65	110	1
0	Galacturonyl	2.7	70	5	95	103	2
20	"	27.0	5	75	95	103	2
40	0	14.4	0	40	90	140	present
0	n-Propyl	3.7	50	0	90	"	"
20	"	28.6	0	72	90	"	"
0	Succinimido	1.8	50	0	90	"	"
20	"	18.2	5	55	90	"	"
20	Sulfadiazine	14.5	5	50	90	"	"
20*	0	6.0	0	28	90	"	"

* Treatment delayed for 3 weeks after infection.

in terms of "chemotherapeutic effectiveness" (the ratio of the average extent of tuberculous involvement in the control and treated groups) it is evident that the efficacy of the combination is greater in every instance than the sum of effects from the individual components. Also the highest percentage incidence of animals free from gross lesions has been realized in the experimental groups treated with the combination, though a very substantial percentage appeared to be free from the disease in the group treated with streptomycin alone at the highest dosage level it has been employed.

It is interesting that sulfadiazine appears to have contributed, if anything, less than any of the sulfones in the combination with streptomycin. The effectiveness of sulfadiazine in the treatment of experimental tuberculosis is also slight compared with the sulfones.^{3,6}

Little can be said at this time concerning the possible mechanisms that may be involved in the mutual potentiation of streptomycin and the sulfones. Our knowledge of the mechanism of antibacterial action of either streptomycin or of the sulfones is fragmentary and inadequate. However, evidence has been accumulating to indicate that bacteria can acquire a high degree of resistance against the antibiotics including streptomycin.⁷⁻⁹ Youmans and Williston¹⁰ have also shown recently that the tubercle bacillus may acquire a resistance to streptomycin, that streptomycin resistant strains are equally virulent in mice, and the animals so infected are refractory to treatment with streptomycin. We had previously shown in this laboratory that prolonged cultivation of

⁶ Smith, C. R., and Oechli, F. W., *Am. Rev. Tub.*, 1945, **52**, 86.

⁷ Miller, C. P., and Bohnhoff, M., *J. Am. Med. Assn.*, 1946, **130**, 435.

⁸ Graessle, O. E., and Frost, B. M., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 171.

⁹ Finland, M., Murray, R., Harris, H. W., Kilham, L., and Meads, M., *J. Am. Med. Assn.*, 1946, **132**, 16.

¹⁰ Youmans, G. P., and Williston, E. H., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 131.

the tubercle bacillus in a medium containing low concentrations of promin resulted in attenuation of virulence.¹¹ It is possible therefore that the beneficial effect of the combined action of the 2 chemotherapeutic agents consists in eliminating or attenuating strains which might acquire a resistance to the antibiotic.

Summary and Conclusions. The chemotherapeutic action of streptomycin used alone or in combination with one of two sulfones or sulfadiazine was studied in experimental tuberculosis in guinea pigs.

Evidence has been obtained to indicate that the therapeutic effect from combined treatment is greater than the sum of effects from the individual components.

The chemotherapeutic effectiveness (ratio of extent of tuberculous involvement in a group of untreated controls and treated

¹¹ Emmart, E. W., and Smith, M. I., *Proc. Soc. Exp. Biol. and Med.*, 1942, **51**, 320.

groups) in a group of guinea pigs treated with 40 mg (40,000 units) streptomycin per kg of body weight daily was 14.4. Previously treatment with 10 to 15 mg per kg per day under similar conditions gave a chemotherapeutic effectiveness of 5.2.

The chemotherapeutic effectiveness of combined therapy with 20 mg streptomycin per kg per day plus 500 mg 4-amino-4'-propylaminodiphenylsulfone per kg per day was 28.6. The chemotherapeutic effectiveness of the sulfone alone was 3.7.

Similar though less marked potentiation was obtained in combined therapy with streptomycin and 4-amino-4'-succinimido-diphenylsulfone. By itself this latter sulfone was less effective than the *n*-propyl derivative.

Combined therapy with streptomycin and sulfadiazine, a substance of doubtful efficacy in experimental tuberculosis, gave inconclusive evidence of potentiation.

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Further Studies on the Testing of Sterility of Concentrated Streptomycin Solutions.

DANIEL LAPEDES, RICHARD DONOVICK, AND GEOFFREY RAKE.

From the Division of Microbiology, The Squibb Institute for Medical Research, New Brunswick, N.J.

The inactivation of streptomycin with semicarbazide, as an aid in testing the sterility of concentrated solutions of streptomycin, was proposed in an earlier paper.¹ A similar method of sterility testing involving the use of hydroxylamine has been described in tentative minimum specifications for streptomycin issued by the Food and Drug Administration.² As is shown in Table I, semicarbazide, as compared to hydroxylamine mol for mol, has the advantage for such sterility test procedures in that the bacteriostatic action of the former is

less than that of the latter for most organisms tested.* If instead of thioglycolate broth, which was used in the present tests, tryptone broth is used, the differences in the bacteriostatic action of the two compounds are even more marked in favor of semicarbazide.¹ The same is true of effects on bacteria in aqueous streptomycin solutions in which the antibacterial power of the carbonyl reagent would be exerted in the suggested sterility tests.

However, recent experiences lead to the

¹ Rake, G., and Donovan, R., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 31.

² *Tentative Minimum Specifications for Streptomycin*, Food and Drug Admin., June 28, 1946.

* Amongst the organisms tested were a number of strains kindly made available to us by Dr. W. A. Randall of the Food and Drug Administration and said to be less susceptible to hydroxylamine than to semicarbazide.

TABLE I.
Comparative Baeteriostatic Action of Semicarbazide-HCl and Hydroxylamine-HCl Against Various Test Organisms in Thioglycolate Broth.

Test organism	M.I.C.*				
	Semicarbazide HCl		Hydroxylamine HCl		Mol ratio SC/HA
	mg/ml	millimols/L	mg/ml	millimols/L	
<i>E. coli</i> SR†	0.60	5.4	0.30	4.3	1.25
<i>K. pneumoniae</i> (A.T.C.C. No. 9997)	0.21	1.9	0.17	2.4	0.79
No. 2411‡	0.15	1.3	0.044	0.63	2.06
No. 2435‡	0.14	1.3	0.039	0.56	2.32
<i>B. brevis</i> §	0.71	6.4	0.18	2.6	2.46
<i>B. circularis</i> §	0.79	7.1	0.20	2.9	2.44
<i>B. subtilis</i> (Peoria)§	0.76	6.8	0.23	3.3	2.06
<i>Pseudomonas aeruginosa</i> §	0.30	2.7	0.17	2.4	1.12
<i>Aerobacter aerogenes</i> §	0.37	3.3	0.25	3.6	0.92
<i>B. subtilis</i> (Merck)§	0.35	3.1	0.13	1.9	1.63
<i>Staphylococcus aureus</i> (Osgood)§	1.16	10.4	0.14	2.0	5.20

* Minimum inhibiting concentration.

† Strain of *E. coli* isolated from solution containing ca. 100,000 units streptomycin/ml.

‡ Psychrophilic organisms isolated from solution containing ca. 100,000 units streptomycin/ml.

§ These strains of bacteria were obtained from the Food and Drug Administration through the kindness of Dr. William A. Randall.

conclusion that the use of either carbonyl reagent can result in false negative results as to the presence of contaminants in concentrated streptomycin solutions. These findings were anticipated somewhat in the earlier paper¹ when it was pointed out that, since carbonyl reagents *per se* are bacteriostatic substances, the procedure suggested (for inactivation of and sterility testing of streptomycin) was valid only to the extent that viable organisms in streptomycin solutions were able to withstand the action of carbonyl reagents.

In recent months it has been noted that certain solutions of streptomycin, containing ca. 100,000 units per ml, on standing at room temperature or at 5°C, became turbid with what appeared to be bacterial growth. While sterility tests according to the procedure described¹ failed to show any growth, simple streaks of these original solutions on yeast beef agar plates yielded one, and in some cases 2, strains of bacteria. One of these was a strain of *Escherichia coli* and 2 others (No. 2411 and No. 2435, possibly identical, although isolated from separate sources) were Gram negative, psychrophilic rods which grew readily at 5° to 25°C but poorly at 37°C.

Pure cultures of these 3 organisms were inoculated into streptomycin solutions containing ca. 30,000 units per ml. The inoculated solutions were divided into several por-

tions, some of which were treated with semicarbazide and potassium acetate as described earlier,¹ while the remainder served as controls. Although the organisms could be readily recovered from the untreated streptomycin solutions, they could not be reisolated from the solutions treated with semicarbazide. Similarly failure resulted from subsequent tests with equivalent amounts of hydroxylamine as the inactivating agent, and, as shown in Table I, all 3 organisms were more sensitive to hydroxylamine than to semicarbazide.

Thus, whereas the use of carbonyl reagents had aided in the demonstration of the presence in streptomycin solutions of certain spore formers,¹ they now played a detrimental role. There still remains a need for a non-germicidal agent which will inactivate streptomycin. Van Dolah and Christenson³ have recently suggested the use of certain oxidizing and reducing agents, such as potassium permanganate and potassium periodate, for the inactivation of streptomycin. However, here again it is likely that, in higher concentrations required for a sterility test, some bacterial destruction would be caused by the reagent before the reaction had gone to completion and before the streptomycin-reagent mixture could be added to test culture media.

³ Van Dolah, R. W., and Christenson, G. L., *Arch. Biochem.*, 1947, **12**, 7.

Further consideration apparently should also be given to the choice of culture medium and the temperature of incubation in sterility testing of streptomycin solutions. Broth containing sodium thioglycolate, because of the ability of the latter to interfere with the action of many antibacterial substances,⁴ is widely used for sterility testing of biological materials. The extent to which this broth interferes with the action of streptomycin⁵ led to its use in a proposed sterility test.¹ In attempts, however, to isolate the streptomycin-resistant *E. coli* strain, referred to above, from inactivated mixtures of streptomycin and carbonyl reagents, it was found that control cultures died out in one week at 37°C. On further examination it appeared that this organism could be carried in thioglycolate broth if daily subcultures were made, but if transfers were attempted after one week's incubation no growth ensued. It was found that this organism could be recovered from this broth through 6 days of incubation but not after the 7th day. Controls in yeast beef broth yielded positive subcultures throughout at least 8 days.

In the sterility testing of many biological materials it is not an uncommon practice to inoculate the material under question into thioglycolate broth, incubate for one week,

and then subculture if there is any doubt whether growth has occurred. Should this be done with a material containing a contaminant such as the *E. coli* strain used here, the subcultures would yield no growth. In this connection we have found that the 2 psychrophils mentioned above do not grow well in thioglycolate broth, but multiply readily in yeast beef broth.

The occurrence of 2 psychrophilic organisms as contaminants in several highly concentrated streptomycin preparations which we have studied demonstrates also the need for incubating streptomycin sterility tests at more than one temperature. We have not yet found any thermophilic organisms occurring as natural contaminants in streptomycin solutions, but this possibility should not be overlooked.

Summary. 1. Further studies with semicarbazide and hydroxylamine confirm earlier findings that for most of the bacterial species tested the former substance mol for mol is less bacteriostatic than the latter. 2. The use of either carbonyl reagent in sterility tests of concentrated streptomycin solutions, however, may lead to false negative findings. 3. Three strains of microorganisms have been isolated from streptomycin solutions which failed to grow out from similar material inactivated with either carbonyl reagent. 4. These organisms isolated from the streptomycin solutions grow poorly in thioglycolate broth, and one species was found to die out in this broth on incubation prolonged beyond 6 days. 5. The demonstration of psychrophilic organisms in streptomycin solutions suggests the need for incubating such sterility tests at more than one temperature.

⁴ a. Eagle, H. J., *Pharmacol.*, 1939, **66**, 436; b. Fildes, P., *Brit. J. Exp. Path.*, 1940, **21**, 67; c. Chow, B. F., and McKee, C. M., *Proc. Soc. Exp. Biol. and Med.*, 1945, **58**, 175; d. Cavalito, C. J., Bailey, J. H., Haskell, T. H., McCormick, J. R., and Warner, W. F., *J. Bact.*, 1945, **50**, 61; e. Geiger, W. B., and Conn, J. E., *J. Am. Chem. Soc.*, 1946, **67**, 112.

⁵ Donovick, R., and Rake, G., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61**, 224.

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Dietary Production of Gastric Ulcers in Rats and Prevention by Tocopherol Administration.

P. L. HARRIS, E. L. HOVE, M. MELLOTT, AND K. HICKMAN.

*From the Laboratories of Distillation Products, Inc., Rochester, N.Y.**

A previous paper¹ from this laboratory reported that α -tocopherol prevented stomach ulcers in rats receiving minimal amounts of vitamin A. The possible explanation advanced to explain this action was based upon the well-known "sparing" of vitamin A by tocopherol, *in vivo*.²

Further experiments were designed to test this explanation and also to determine whether tocopherols would be beneficial in preventing stomach ulcers caused by dietary restrictions other than vitamin A.

In a preliminary experiment 40 weanling male rats were placed on a diet (USP XII, with olive oil), low in vitamins A and E, for 2 weeks. At this time supplementation was begun of all rats with 300 units of vitamin A-acetate daily. Half of the rats were also given 0.5 mg of *d*, α -tocopherol daily. After 7 weeks the animals were killed and examined for gastric lesions. Fifty per cent of the rats on the high-A, low-E diet had one or more ulcers in the fore-stomach, while none of the rats receiving vitamin E had lesions.

In a second experiment 170 male weanling rats were completely depleted of vitamin A in the usual manner. The diets used were based upon the USP vitamin A-test diet, but with variable amounts and kinds of fat. These variations are indicated in Table I. At the time of depletion 3 or 4 rats from each group

* Communication No. 108.

¹ Jensen, J. L., *Science*, 1946, **103**, 586.

² Hickman, K. C. D., Kaley, M. W., and Harris, P. L., *J. Biol. Chem.*, 1944, **152**, 303; Harris, P. L., Kaley, M. W., and Hickman, K. C. D., *J. Biol. Chem.*, 1944, **152**, 313; Hickman, K. C. D., Kaley, M. W., and Harris, P. L., *J. Biol. Chem.*, 1944, **152**, 321.

TABLE I.
Effect of Tocopherol and Dietary Fat on Stomach Lesions of Rats After Cure of Vitamin A Deficiency with High Doses of Vitamin A (10 Rats per Group).

Group	Diet—Fat	Avg depletion time (days)	Supplement after depletion		Avg wt gain 48 days	Incidence of gastric ulcers, %	Avg severity of ulcers graded 1-10
			Units Vit. A per day	Tocopherol per day			
I	5% olive oil	62	30		96.4	50	4.6
I ^A	" "	62	30	.5 mg d ₁₂	94.0	0	—
I ^B	" "	62	2		56.9	60	5.1
I ^C	" "	62	2	" " "	63.1	0	—
I ^D	" "	62	2	" " phosphate inj.	59.8	50	4.3
II	10% " "	65	30		97.3	0	—
II ^A	" "	65	30	.5 mg d ₁₂	100.7	0	—
III	5% stripped corn oil	60	30		99.1	30	3.7
III ^A	" "	60	30	" " "	101.9	0	—
IV	10% " "	61	30	" " "	98.4	20	2.0
IV ^A	" "	61	30	" " "	100.2	0	—
V	Fat-low	51	30		62.5	80	6.1
V ^A	" "	51	30	" " "	57.8	80	5.7
V ^B	" " + 100 mg sesame oil	51	30	" " "	77.1	30	3.9
V ^C	" " " "	51	30	" " "	83.8	10	2.3

were examined, but no gross lesions were present. It is evident that vitamin A-deficiency *per se*, does not produce ulcers. The depleted animals were placed on a daily supplement of 2 or 30 units of vitamin A-ester concentrate, dissolved in olive oil, with or without 0.5 mg α -tocopherol. Half of the rats on the low-fat diet were supplemented daily with 100 mg of sesame oil to supply adequate linoleic acid.

Table I shows the data on gain in body weight and incidence and severity of rumen lesions after a 7-week experimental period.

It is highly significant that vitamin A even in high doses did not prevent ulcer development. This indicates that the action of tocopherol cannot be explained simply as a sparing of vitamin A in the gastrointestinal tract. However, the fact that injections of tocopheryl phosphate did not prevent the lesions is presumptive evidence that the tocopherol effect is localized in the stomach. It is interesting to reflect at this time that in all vitamin A bioassays which have been conducted without providing adequate vitamin E to the animals during the depletion or curative periods, 50% or more of the animals used probably had gaping lesions in their stomach. These gastric ulcers certainly were an important factor in causing some of the disturbingly wide variations in growth response observed in vitamin A bioassays.

The influence of fat in the diet is interesting. The lesions occurred on the 5% level of olive oil or stripped (E-free) corn oil; but on the 10% oil level the lesions were absent or reduced in number even without tocopherol supplements. On the low-fat diet a very high incidence and severity of lesions was noted and tocopherol at the levels used had no beneficial effects.

Since fat in the diet is a significant factor in the prevention of rumen lesions, it was of interest to examine the stomachs of rats deficient only in essential fat acids. The fat-low diet used in the preceding experiment contained 56% corn starch which probably supplied about 0.6% fat as a constituent.³

Thus the diet contained about 0.3 to 0.4% fat which furnished at least 10 mg linoleic acid daily to each animal.⁴

In the present experiment, weanling rats were placed on a fat-free diet of the following percentage composition: Casein (Labco) 20, sucrose 76, and salt mixture, USP No. 2, 4. B-complex vitamins were added to the casein to furnish 10 γ of thiamin, riboflavin, and pyridoxine, 25 γ calcium pantothenate, 1 mg choline chloride, and 0.1 mg *i*-inositol per gram of ration. Fifty units of vitamin A-acetate and 5 units of vitamin D were given daily in a propylene glycol solution.

After 7 weeks depletion the rats were divided into groups of 6 each and placed on experiment with and without 20 mg of ethyl linoleate also with and without 0.5 mg tocopherol daily. After 6 weeks the stomachs of the rats were examined for lesions. The results are shown in Table II. Hyperplasia of the juncture (or ridge) separating the glandular portion from the rumen, usually precedes or accompanies hyperplastic ulcers in the rumen. The incidence of such juncture hyperplasia has been indicated, as well as the incidence of ulcers.

The data in Table II show that rats deficient in essential fatty acids do not have stomach lesions. However, half of the rats receiving 20 mg of linoleate showed typical ulcers. Juncture hyperplasia was marked in this group. Either α - or γ -tocopherol prevented the lesions.

It was of further interest to determine whether ulcers produced by dietary restrictions other than vitamin A or essential fatty acids would also be prevented by tocopherol.

Male albino weanling rats were placed upon a pyridoxine-deficient ration for 6 weeks. The percentage composition of the ration was: Casein (Labco) 20, sucrose 71, salt mixture, USP No. 2, 4, lard 4, and cod liver oil 1. B-complex vitamins were added to the casein to furnish 10 γ thiamin and riboflavin, 25 γ calcium pantothenate, 1 mg choline chloride, and 50 γ of vitamin K per gram ration.

³ Taylor, T. C., and Nelson, J. M., *J. Am. Chem. Soc.*, 1920, 42, 1726.

⁴ Taylor, T. C., and Lehrman, L., *J. Am. Chem. Soc.*, 1926, 48, 1739.

TABLE II.
Effect of Tocopherol and Methyl Linoleate on Development of Gastric Lesions in Rats Deficient in Essential Fatty Acids.*

Trial	Daily supplement		Incidence of gastric lesions	
	Methyl linoleate (mg)	d, α -tocopherol (mg)	Ulcers (%)	Juncture hyperplasia (%)
I	0	0	0	0
	0	0.5	0	17
	20.0	0	50	67
	20.0	0.5	0	0
	20.0	0.5†	0	0
II	0	0	0	33
	20.0	0	0	83
	20.0	0.5	0	17

* 6 animals per group.

† γ -tocopherol.

TABLE III.
Effect of Tocopherol in Preventing Development of Rumen Lesions in Rats Recovering from Pyridoxine Deficiency.

Pyridoxine (γ daily)	Mg α -tocopherol daily	No. of rats	Avg wt gain in 6 wk (g \pm S.D.)	Incidence of rumen lesions (%)
0	0	7	4.3	0
	1	7	7.1	0
1.0	0	4	51.5 \pm 6.3	50
	1	4	53.5 \pm 4.9	0
2.0	0	7	71.4 \pm 7.6	57
	1	7	82.7 \pm 8.3	0
4.0	0	7	91.3 \pm 5.0	29
	1	7	105.8 \pm 4.8	0
8.0	0	6	124.0 \pm 7.7	0
	1	5	120.0 \pm 6.0	0

After 6 weeks depletion, the rats were fed various levels of pyridoxine with and without tocopherol. Pyridoxine (free-base) was prepared from synthetic pyridoxine-hydrochloride (Merck) and given in propylene glycol solution from a calibrated dropper.

The resulting gains in body weight during the subsequent 6 weeks and the incidence of rumen lesions at this time are summarized in Table III. These data indicate that pyridoxine deficiency in itself does not result in rumen lesions. However, when suboptimal doses of pyridoxine are fed, lesions do occur but are preventable by α -tocopherol. Higher doses of pyridoxine maintain normal stomachs in the rats even without tocopherol.

Discussion. It is evident that gastric lesions can be induced by a variety of dietary deficiencies when these are combined with low intakes of vitamin E. Severe tocopherol de-

ficiency uncomplicated by deficiencies of other essentials does not induce ulcers. Hundreds of vitamin E-deficient female rats with evidence of resorption gestations have been free of stomach lesions. Furthermore, uncomplicated deficiencies of vitamin A, or of pyridoxine, or of essential fatty acids do not induce ulcers. Ulcers are formed only in rats which have stopped gaining weight as a result of any of these deficiencies and which then have been given the missing factor but without supplying tocopherols at the same time. Weight gain is resumed when vitamin A, pyridoxine, or linoleate, respectively, is supplied and it is during this period that stomach lesions appear unless sufficient tocopherol is present in the gastrointestinal tract. Tocopherols injected as the tocopheryl phosphate during the experimental period fail to prevent ulcer formation. Consequently,

the evidence points to a mechanistic explanation for lesion formation. Functionally, the epithelium of the forestomach of the rat becomes abnormal due to deficiencies of either vitamin A, or pyridoxine, or essential fatty acids. Anatomically there are no lesions because of the gradual adaptation of the organism as a whole to a low level of metabolic activity: food intake decreases, weight gain ceases, and probably gastric secretion of pepsin and HCl is diminished. However, administration of the missing essential factor induces food intake and resumption of weight gain. Unless adequate tocopherol is also administered the functionally abnormal epithelium of the forestomach cannot cope with the increased acid-pepsin medium with which it is in contact and as a result lesions are produced.⁵ Whether tocopherols prevent the erosion of the stomach wall by specifically inhibiting enzyme activity or more fundamentally by increasing epithelial resistance perhaps by inducing greater mucous secretion or improving blood circulation in the mucosa⁶ is not yet known.

The role of dietary fat in the etiology and treatment of gastric lesions is also undetermined. In our experiments an increase of fat (olive oil or vitamin E-free corn oil) from 5 to 10% in the diet reduced the incidence and severity of ulcers formed. Li and Freeman⁷ also found a higher incidence of peptic ulcers in dogs on a low-fat (lard) than on

a high-fat diet. However, Matzner and co-workers⁸ reported their highest incidence and most severe stomach lesions in rats maintained on relatively high fat (butter or lard) intakes.

It is emphasized that no attempt should be made at this time to draw a parallel between these gastric ulcers in rats and those in humans. Much more must be learned concerning the etiology and pathology of the lesions in the 2 species before generalizations regarding treatment with fat or tocopherols can be made. The level of protein intake is also important and data showing a protein-tocopherol interrelationship as measured by ulcer production and cure is being prepared for publication.

Conclusions. 1. Ulcers in the forestomach were induced in rats: (a) Recovering from vitamin A-deficiency with 2, 30, or 300 units vit. A daily. (b) Recovering from a vit. B₆ deficiency with suboptimal amounts (1, 2, or 4 γ daily) of pyridoxine. (c) Recovering from an essential fatty acid deficiency with 20 mg of linoleate daily.

2. Deficiencies of these 3 essentials did not, in themselves, result in lesions. The ulcers occurred only during the curative period in which the specific nutrient was fed.

3. α -Tocopherol fed daily to the rats during the cure of the specific deficiency gave complete protection. γ -Tocopherol orally was also effective, but α -tocopheryl phosphate injected was not.

4. The amount of fat in the diet was related to the incidence of stomach ulcers in the vitamin A experiments. Ten per cent gave good protection. Five per cent allowed moderately severe lesions, which were preventable by tocopherol. Fat-low diets gave very severe lesions not preventable by the level of tocopherol fed, 0.5 mg daily.

⁵ Matzner, M. J., Windmer, C., Sobel, A. E., and Polayes, S. H., *Proc. Soc. Exp. Biol. and Med.*, 1936, **34**, 243.

⁶ Bachrach, W. H., Grossman, M. I., and Ivy, A. C., *Gastroenterology*, 1946, **6**, 563.

⁷ Li, T., and Freeman, S., *Gastroenterology*, 1946, **6**, 140.

⁸ Matzner, M. J., Windmer, C., and Sobel, A. E., *Am. J. Dig. Dis.*, 1938, **5**, 1.

Nature of Renal Changes in Acute Choline Deficiency.

F. I. DESSAU AND J. J. OLESON. (Introduced by Y. Subbarow.)

From the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N.Y.

Changes in the kidneys of acutely choline-deficient young rats, referred to as hemorrhagic degeneration of the kidneys were described by Griffith and Wade,¹ and later by Györgyi and Goldblatt,² Christensen,³ Engel and Salmon⁴ and others. The most careful histological study by Christensen showed the quite secondary role of hemorrhage in this syndrome, but left open the question of interdependence of tubular degeneration and vascular congestion.

Experimental. Young rats, 20 to 21 days old and weighing about 35 g, were placed in individual cages and fed water and the following diet *ad libitum*:

Basal diet:

Sucrose	%
Alc. Ext. Casein	65
Salts No. 2	15
Lard	5
Gelatin	10
Corn Oil	3
	2

with the addition of the following vitamins/kg:

Thiamin chloride	10	mg
Pyridoxine hydrochloride	10	"
Calcium pantothenate	20	"
Niacin	10	"
Para-aminobenzoic acid	20	"
Riboflavin	10	"
Inositol	50	"
Biotin	0.2	"
Vit. E	25	"
Menadione	5	"
Vit. A	20,000	units
" D	2,000	"

It was determined by observation that under our conditions the renal syndrome began to appear at about the fifth day. Thus most experiments were terminated on the

seventh day since by this time all the rats would show marked renal hyperemia.

A detailed examination of the kidneys for structural changes due to this deficiency, led to the following conclusions as to the nature and sequence of these changes:

(1) Both grossly and microscopically the change observed first is venous stasis resulting in extreme cyanosis of the outer cortex.

(2) Absence of red blood cells from the lumen of the tubules is evidence of lack of hemorrhage.

(3) Epithelial degeneration and necrosis, limited to the tubules occupying the outer cortex, are secondary to the vascular disturbance.

(4) The lack of glomerular involvement, in sharp contrast to the marked tubular injury, proves interference with venous outflow rather than disturbance of arterial supply.

The outlined sequence of events of the renal syndrome is observed in reversed order in the phase of recovery, with venous stasis disappearing most rapidly, epithelial regeneration following considerably later, and hyaline casts, distended tubules and small scars forming the last residues of the disturbance. That interference with venous outflow is involved, finds support in our observations, that ligation of the renal vein in rats results in lack of damage to the glomeruli while there is marked injury to the tubular epithelium.

Experimental evidence of the priority of vascular disorder over epithelial injury is suggested by the protective effect of renal decapsulation. This operation was performed under ether anesthesia on the left side only, the right kidney serving as a control. In one group of experimental animals an incision was made on both sides but decapsulation of the kidney was performed on the left side only. The operation took place

¹ Griffith, W. H., *Biol. Symposia*, 1941, **5**, 193;
Griffith, W. H., and Wade, N. J., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 188.

² Györgyi, P., and Goldblatt, H., *J. Exp. Med.*, 1940, **72**, 1.

³ Christensen, K., *Arch. Path.*, 1942, **34**, 633.

⁴ Engel, R. W., and Salmon, W. D., *J. Nutrition*, 1941, **22**, 109.

on the day that the rats were put on the choline-deficient diet. This operation gave protection to the decapsulated kidney in proportion to the completeness of the operation. In 19 out of 34 animals there was complete protection from the renal syndrome of acute choline deficiency on the operated side. This protection seems to be in abeyance if the operation is not performed well in advance of the acute disturbance, since if the decapsulation is not done until the fifth day, no protection is observed.

Wolbach⁵ has suggested the possibility that a neurovascular mechanism might be the basis of the renal syndrome observed in acute-

⁵ Wolbach, S. B., and Besser, O. A., *Physiol. Rev.*, 1942, 22, 233.

ly choline-deficient rats. There is support for such an assumption in our findings that subcutaneous administration of atropine seems to inhibit the renal syndrome. When rats were treated with 1 mg atropine sulfate daily, from the day they were started on the choline-deficient diet, prevention of the renal syndrome was observed in almost half of the cases. In these preliminary experiments, which lasted 7 days, 10 of 21 choline-deficient young rats failed to show the "hemorrhagic renal syndrome."

Summary and Conclusion. Morphological and experimental studies suggest a vascular disorder to be the primary injury observed in the kidney of the acutely choline-deficient rat.

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Reticulo-Endothelial Immune Serum (REIS). VI. Production of Potent Serum by Anamnestic Reaction.*

LUDWIK ANIGSTEIN, DOROTHY M. WHITNEY, C. M. POMERAT, AND MARY FAITH ORR.

From the Departments of Preventive Medicine and of Anatomy, University of Texas, Medical Branch, Galveston.

The development of antiorgan sera has been reported in studies from our laboratories.¹ Whereas in our original work² we followed the method of Soviet workers,³ some modifications in the production of REIS were introduced in the present study. It was thought that an accumulation of antibodies in the recipient host would be enhanced by a slow process of immunization rather than by the rapid method of using massive doses.⁴

In the preparation of antiguinea pig and of antirat sera, constant amounts of tissue,

400 mg of spleen plus 100 mg of bone marrow, suspended in 6 ml of saline were administered to the rabbit. Intravenous antigen injections of 0.25, 0.5, 0.5, and 0.75 ml were given at 4-day intervals, and the same amounts were given by mouth, the total being approximately 133 mg of spleen and 33 mg of bone marrow by each route within 12 days.

Complement-fixation titres illustrate the high values of antirat and antiguinea pig sera following low antigenic dosages.

The achievement and maintenance of adequate potency of the serum was one of the main objectives. Attempts were made, therefore, to provoke an increased antibody production in previously immunized animals by their anamnestic response to a renewed contact with the antigen. A single intravenous injection of the same type of antigen 30-40 days following the last injected dose was administered to rabbits.

* Supported by a grant from the Lilly Research Laboratories.

¹ Pomerat, C. M., *Phi Beta Pi Quart.*, 1945, 42, 203.

² Pomerat, C. M., and Anigstein, L., *Tex. Rep. Biol. and Med.*, 1945, 3, 122.

³ Marchuk, P. D., *Am. Rev. Sov. Med.*, 1943, 1, 113.

⁴ Strans, R., Ruujavac, M., Zaitlin, R., Duboff, G., and Swerdlow, H., *II. J. Immun.*, 1946, 56, 155.

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Experimental. Young rats, 20 to 21 days old and weighing about 35 g, were placed in individual cages and fed water and the following diet *ad libitum*:

Basal diet:

Sucrose	65
Alc. Ext. Casein	15
Salts No. 2	5
Lard	10
Gelatin	3
Corn Oil	2

with the addition of the following vitamins/kg:

Thiamin chloride	10	mg
Pyridoxine hydrochloride	10	"
Calcium pantothenate	20	"
Niacin	10	"
Para-aminobenzoic acid	20	"
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(4) The lack of glomerular involvement, in sharp contrast to the marked tubular injury, proves interference with venous outflow rather than disturbance of arterial supply.

The outlined sequence of events of the renal syndrome is observed in reversed order in the phase of recovery, with venous stasis disappearing most rapidly, epithelial regeneration following considerably later, and hyaline casts, distended tubules and small scars forming the last residues of the disturbance. That interference with venous outflow is involved, finds support in our observations, that ligation of the renal vein in rats results in lack of damage to the glomeruli while there is marked injury to the tubular epithelium.

Experimental evidence of the priority of vascular disorder over epithelial injury is suggested by the protective effect of renal decapsulation. This operation was performed under ether anesthesia on the left side only, the right kidney serving as a control. In one group of experimental animals an incision was made on both sides but decapsulation of the kidney was performed on the left side only. The operation took place

¹ Griffith, W. H., *Biol. Symposia*, 1941, **5**, 193; Griffith, W. H., and Wade, N. J., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 188.

² Györgyi, P., and Goldblatt, H., *J. Exp. Med.*, 1940, **72**, 1.

³ Christensen, K., *Arch. Path.*, 1942, **34**, 633.

⁴ Engel, R. W., and Salmon, W. D., *J. Nutrition*, 1941, **22**, 109.

Effects of Benadryl on Anaphylactic and Histamine Shock in Rabbits and Guinea Pigs.

BERRY CAMPBELL, IVAN D. BARONOFKY,* AND ROBERT A. GOOD.

From the Departments of Anatomy and Surgery, The Medical School, University of Minnesota.

Recent work^{1,2} suggests that the mechanism of anaphylactic shock is best explained with the inclusion of a stage at which histamine is released. On the assumption that the release of histamine is the factor leading to shock and death, therapeutics of anaphylactic and allergic states has turned to β -dimethylaminoethyl benzhydryl ether hydrochloride ("Benadryl") and related drugs.^{3,4} Success in this has been offered as further proof of the thesis that histamine intoxication is the chief untoward effect of anaphylaxis. Protection against anaphylaxis by benadryl was reported in experiments on passive sensitization of guinea pigs and intravenous administration of the antigen.

The experiments here reported resulted from a search for the antianaphylactic effects of benadryl. In the 2 species studied, one might expect that if the principal symptoms of shock and the cause of death attendant on anaphylactic shock were ascribable to the release of histamine, the amelioration of anaphylactic shock by benadryl would parallel its effects on histamine shock. That such is not the case is shown by the experiments which follow; the implications of these findings will be discussed.

Experiment I. To test the effects of benadryl on histamine shock in rabbits, 25 animals were employed, 12 of which received premedication with benadryl. The animals fell into 3 groups, each containing rabbits of similar size and history and each being di-

vided into experimental and control groups. Benadryl was administered in divided doses totaling 10 mg per kg or in single doses of 4 mg per kg. Striking protection at all the dose levels is demonstrated in Table I.

Experiment II. To test the effects of benadryl on anaphylactic shock in rabbits, 23 animals were sensitized to egg white. This was accomplished by injecting egg white on alternate days in the following dosages: 1 cc, i.v.: 0.5 cc, i.v.; and 1 cc, i.m. The rabbits were used between 3 and 5 weeks following the first dose. Group A consisted of 10 rabbits so sensitized, half of which were premedicated with 5 mg of benadryl per kg 25 minutes before injection of the antigen. The 5 rabbits not treated with benadryl were all severely shocked, and 4 of 5 died. Those premedicated were similarly shocked, and none of the 5 animals recovered. In group B, benadryl (5 mg per kg in 3 animals; 10 mg per kg in 3) was administered 24 hours previously, with a second dose 20 minutes before the antigen. No difference in the effects of a smaller dose of antigen (0.6 cc) was observed between experimental and control animals.

Experiment III. To test the effects of benadryl on anaphylactic shock in the actively sensitized guinea pig, each of 11 animals was sensitized by a single intraperitoneal injection of 5 cc 25% egg white in saline. Twenty days later 7 of these received 10 mg of benadryl per kg administered intraperitoneally. Thirty minutes later 5 of the premedicated animals and 4 controls were injected intraperitoneally with 0.75 cc of egg white. All animals showed severe shock; 3 of 4 control, and 3 of 5 treated, guinea pigs died. Two animals given only benadryl showed no ill effects.

Discussion. Experiment I indicates that benadryl is, under the conditions of the ex-

* Trainee of the National Cancer Institute.

¹ Dragstedt, C. A., *J. Allergy*, 1945, **16**, 69.

² Code, C. F., *Proc. Staff Meetings Mayo Clin.*, 1945, **20**, 439.

³ Loew, E. K., and Kaiser, M. E., *Proc. Soc. Exp. Biol. and Med.*, 1945, **58**, 235.

⁴ Friedlaender, S., Feinberg, S., and Feinberg, A. R., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 65.

TABLE I.
Showing Comparative Values of REIS Potency in a Unit Experiment.

Rabbit No.	Antigen source	REIS complement-fixation titers		Spleen explant outgrowth in REIS 1:4	
		Primary	Anamnestic	Primary	Anamnestic
68	Chick	1:60	X	+	X
69	"	1:40	1:640	++	+
70	Guinea pig	1:640	1:1280	+++	+
71	" "	1:1000	1:2000	+	0
72	Rat	1:1000	X	0	X
73	"	1:320	1:160	+	++

X Animal sacrificed.

0 No outgrowth—complete inhibition.

+

The serum of rabbit 69 (Table I), treated with chick antigen, primarily showed a titre of only 1:40. This rabbit was given a booster dose 30 days later of 1:25 ml suspension of 400 mg of chick spleen and 100 mg of bone marrow in 6 ml saline. The animal was bled 9 days later and at this time the "anamnestic" serum, as compared with the "primary," showed a titre for complement fixing antibodies of 1:640. A 100% rise in the titre was obtained in 2 other rabbits previously treated with guinea pig antigen. On rare occasions there was no response to the booster method (rabbit 73).

The potency of the serum was also evaluated by the tissue culture assay method. It was previously suggested⁵ that it is possible to measure the action of antiorgan preparations by the degree of inhibition of cellular outgrowth from explants in a medium containing various concentrations of REIS. The outgrowth equivalent to that of untreated explants was designated as + + + +. When the serum of rabbit 69 (primary titre 1:40)

was tested, the outgrowth of 15- to 17-day chick embryonic spleen fragments was moderately good (+ +), showing weak inhibitory effect of the serum. However, the serum of the same rabbit, after the anamnestic reaction, reaching the titre of 1:640 almost completely inhibited the outgrowth. Marked clumping of outwandering cells under the influence of the serum of increased potency at dilutions of 1:4, 1:8, and 1:16 was noted. The anamnestic serum totally inhibited the outgrowth in concentrations of 1:4, 1:8, 1:16, 1:32 as compared with a slight inhibition of the primary serum.

Summary. In the preparation of antiorgan sera the administration of small amounts of antigen (spleen and bone marrow) was found more effective than the rapid immunization of rabbits with massive antigen dosage. A single injection of the antigen 30-40 days after the last dose gives rise to an anamnestic response of the host and to a serum of higher potency. This can be evaluated by complement fixation titres and by the outgrowth of tissue explants exposed to the action of the "anamnestic" serum.

⁵ Pomerat, C. M., and Anigstein, L., *Science*, 1944, 100, 456.

TABLE II.
Effects of Benadryl on Anaphylactic Shock in Rabbits.

Group	No.	Wt, kg	Sensitive	Premedication	Shock dose	Effect
A	1	2.0	Yes	None	1.0 cc egg white	4
	2	1.8	"	"	"	4
	3	1.8	"	"	"	4
	4	2.0	"	"	"	4
	5	1.8	"	"	"	3
	6	2.3	"	Benadryl, i.p., 5 mg per kg 25 min before injection with antigen	"	4
	7	1.8	"	"	"	4
	8	1.8	"	"	"	4
	9	2.0	"	"	"	4
	10	2.3	"	"	"	4
B	11	1.8	"	Benadryl, i.p., 5 mg per kg 24 hr before shock, plus 5 mg per kg 20 min before antigen.	0.6 cc	3
	12	1.8	"	"	"	2
	13	1.8	"	"	"	3
	14	1.8	"	Benadryl, i.p., 10 mg per kg 24 hr before shock, plus 5 mg per kg 20 min before antigen.	"	4
	15	1.8	"	"	"	3
	16	1.8	"	"	"	4
	17	1.8	"	None	"	3
	18	1.8	"	"	"	4
	19	1.8	"	"	1.0 cc	3
	20	1.8	"	"	"	3
	21	1.8	"	"	"	2
	22	1.8	"	"	"	4
	23	1.8	"	"	"	2

TABLE III.
Effects of Benadryl on Anaphylactic Shock in Actively Sensitized Guinea Pigs.

No.	Wt, g	Premedication	Antigen	Results
1	350-400	None	0.75 cc egg white, i.p.	Died in 20 min.
2	"	"	"	" " 30 "
3	"	"	"	" " 35 "
4	"	"	"	Survived prolonged severe shock
5	"	10 mg per kg 30 min before antigen	"	Died in 30 min.
6	"	"	"	" " 35 "
7	"	"	"	" " 40 "
8	"	"	"	Survived prolonged severe shock
9	"	"	"	" " " "
10	"	"	None	No ill effects
11	"	"	"	" " "

tection against histamine shock in rabbits. 2. Benadryl did not protect against anaphylactic shock in rabbits sensitized to egg white. 3. Benadryl did not protect guinea

pigs actively sensitized to egg white against anaphylaxis induced by intraperitoneal administration of the antigen under the dosage conditions employed.

TABLE I.
Effects of Benadryl on Histamine Shock in Rabbits.

Group	No.	Wt, kg	Premedication	Histamine dose	Effect*
A	1	1.8	None	2.0 mg hist. phosph.	4
	2	1.8	"	"	4
	3	1.8	"	"	4
	4	1.8	"	"	4
	5	1.8	"	"	3
	6	1.8	Benadryl, i.p., 5 mg per kg 4	"	1
	7	1.8	hr before hist., plus 5 mg	"	0
	8	1.8	per kg 15 min before hist.	"	0
	9	1.8		Died before hist. was admin.	
B	10	2.5	None	2.75 mg hist. phosph.	2
	11	3.4	"	4.13	4
	12	3.0	"	3.44	3
	13	3.0	"	2.75	4
	14	3.0	Benadryl, i.p., 4 mg per kg,	5.50	3
	15	3.2	15 min. before hist.	"	0
	16	3.0	"	"	0
	17	3.6	"	"	0
C	18	2.3	None	2.10	3
	19	2.3	"	"	4
	20	2.3	"	"	4
	21	2.3	"	"	3
	22	2.3	Benadryl, i.p., 4 mg per kg,	2.10	2
	23	2.5	15 min before hist.	"	0
	24	2.3	"	"	0
	25	2.3	"	"	2

* Four grades of shock were as follows: 1, signs of collapse but animal able to move when stimulated; 2, resting in normal position but unable to move; 3, prostrate position or on side; 4, dead of shock.

periment, an effective antagonist of histamine. This means either that it opposes histamine in its effect on the shock organs or that it acts chemically to remove or neutralize histamine. The present experiments do not differentiate between these 2 possible modes of action and the reader is referred to the recent discussion of Code.² The lack of antianaphylactic effect of benadryl shown in Experiment II, even in doses which were highly effective against histamine, indicates either that the histamine released upon anaphylactic shock is not vulnerable to this antihistamine drug, or that some other factor is involved in the shock and death caused by the antigen. As the most recent demonstrations of the antianaphylactic nature of these drugs have been made on guinea pigs, Experiment III on that species was set up (Table III). The 5 experimental animals all showed severe shock, though treated with

10 mg per kg of benadryl. Our results seem at direct variance with those of Loew and Kaiser³ and Friedlaender, Feinberg, and Feinberg,⁴ for they conclude that benadryl offers marked protection to anaphylaxis in the guinea pig. However, it is important to note that their animals were passively sensitized and that they administered the antigen intravenously. While the shock states which they observed were primarily pulmonary in nature, in our animals, in which the antigen was administered by a different route, the shock developed more slowly and was characterized principally by prostration with few respiratory symptoms. Similar alteration of anaphylactic shock with routes of administration other than intravenous have been demonstrated by Williamson.⁵

Summary. 1. Benadryl offers effective pro-

⁵ Williamson, R., *J. Hyg.*, 1936, 30, 588.

that the cholesterol levels were significantly lowered, the hens were sacrificed and blood collected for lipid analysis. The aorta, about a quarter gram of heart muscle and a similar mass of liver were individually and accurately weighed, then macerated with sand and 1 to 3 ether alcohol solution. The extract of each organ was analyzed and the amounts of total cholesterol and cholesterol esters in 100 g of each tissue calculated. Twenty old hens fed as simultaneous controls were also sacrificed and the blood and tissues likewise treated. The results are set down as con-

trols in Table I.

It is evident that the total blood cholesterol and cholesterol ester levels definitely decreased and the phospholipids rose after the old hens had been given 0.5 g methionine daily for about 3 weeks. After 5 to 7 weeks treatment, significantly lower levels of the blood cholesterol and cholesterol esters were established, and the aorta, heart, and liver showed significantly lower lipid levels than those of the control series. Methionine therefore, seems to act as a decholesterolizing agent in old hens.

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Fibrinogenolytic Demonstration of Activation and Inhibition of Tryptase in Plasma Protein Fraction-I ("Antihemophilic Globulin").*

JOHN H. FERGUSON, BURTON L. TRAVIS, AND EARL B. GERHEIM.

From the Department of Physiology, University of North Carolina, Chapel Hill, N.C.

Previous experience has shown the superiority of lysis of fibrinogen¹ over fibrinolysis² in the assay of tryptic enzymes. Recent revival of the idea³ that the natural blood-clotting system involves the same protease (serum-tryptase) that is responsible for fibrinolysis⁴ and other proteolytic phenomena is based upon experimental analogies with crystalline pancreatic trypsin. The ability of trypsin to restore or improve the clotting of hemophilic blood *in vitro*⁵ and *in vivo*⁶ suggests a plasma tryptase deficiency

in hemophilia, for which not-altogether-conclusive evidence is afforded by data^{7,8} on proteases demonstrable by the Delezenne and Pozerski (1903) chloroform-activation method, and by similar finding⁹ of such proteases in certain "globulin" plasma protein fractions, which the Harvard investigators¹⁰ have developed in application to the clinical treatment of hemophilia. The present observations employ a fibrinogenolytic reaction¹ to establish some fundamental considerations with respect to the development of "tryptase" activity in fibrinogen-rich human plasma fractions, as compared with similar materials of bovine plasma origin.

Reagents. 1. H.F.: human plasma Fraction I, courtesy of Drs. Cohn, Minot, Edsall, and colleagues (Harvard Medical School):¹⁰⁻¹²

* This work is the first of a series of investigations on "Enzymes and enzyme-inhibitors, in relation to blood coagulation and hemorrhagic diseases," aided by a grant from the John and Mary R. Markle Foundation.

¹ Ferguson, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1943, **52**, 243.

² Ferguson, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1942, **51**, 73.

³ Ferguson, J. H., *Science*, 1943, **97**, 319.

⁴ Nolf, P., *Medicine*, 1938, **17**, 381.

⁵ Ferguson, J. H., *Am. J. Physiol.*, 1939, **126**, 669.

⁶ Tagnon, H. J., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 45.

⁷ Feissly, R., *Schweiz. Med. Wschr.*, 1942, **72**, 648.

⁸ Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Invest.*, 1943, **22**, 127.

⁹ Kaplau, M. H., Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Invest.*, 1942, **21**, 533.

¹⁰ Lewis, J. H., Tagnon, H. J., Davidson, C. S., Minot, G. R., and Taylor, F. H. L., *Blood*, 1946, **1**, 166.

Methionine Decholesterolization in Old Hens.*†

GEORGE R. HERRMANN.

From the University of Texas, Medical Branch, Galveston, Texas.

Choline has been shown in our previous studies to have a decholesterolizing effect in old hens.¹ Methionine has been found to take part in a reversible reaction with choline in the animal body² and to substitute for choline³ and be effective in lipid metabolism disturbances in the liver of mammals.^{4,5} It therefore seemed logical to study methionine, just as we have choline and inositol, as a possible decholesterolizing agent in old hens with hypercholesterosis.

Forty-four 2-year-old white leghorn hens were individually caged and fed the standard Texas A & M laying mash. The initial blood lipid levels were determined by the

methods of Bloor⁶ for total cholesterol, Bloor and Knudson⁷ for cholesterol esters, and King⁸ for total and inorganic phosphorus.

Methionine, 0.5 g, was placed daily in the gullet of each of 24 of the old hens. One hen died of liver disease. After feeding the methionine for 18 to 29 days, the hens were bled a second time and the blood lipids were again determined, but, as shown in Table I, very little decholesterolization had occurred. The administration of methionine was continued, third bleedings were made after 36 to 51 days, and the bloods chemically analyzed as before.

When random samples of blood showed

TABLE I.
Effect of Methionine on Cholesterol in Blood and Tissue of Old Hens.

	Blood cholesterol, mg/100 ml		Lipid Phosphorus, mg/100 ml	Tissue cholesterol, mg/100 g					
				Aorta		Heart		Liver	
	Total	Esters		Total	Esters	Total	Esters	Total	Esters
Controls									
20 old hens	195	147	10.7	253	188	233	172	303	223
S.D.*	±18	±20	±2	±50	±47	±48	±36	±50	±38
23 old hens									
1st bleeding	228	171	11.2						
S.D.	±33	±23	±2.3						
Methionine									
2nd bleeding	171	126	14.3						
18-29 days S.D.*	±29	±28	±2.4						
3rd bleeding	138	95	16.6	156	102	135	91	276	169
36-57 days S.D.*	±23	±24	±4	±21	±21	±19	±13	±94	±63

* S.D.—Standard Deviation.

* With the technical assistance of Anna H. Williams, Betty Ann Bied, John E. Prewett, H. Tom Leigh, and George W. Reimer.

† Methionine was generously supplied by John Wyeth Company, Philadelphia, and the study was, in part, supported by a grant from the Medical Research Department of the Winthrop Chemical Company.

¹ Herrmann, G. R., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61**, 229, 302; **63**, 436.

² Simmonds, S., and du Vigneaud, V., *J. Biol. Chem.*, 1942, **146**, 685.

³ Griffith, W. H., and Wade, N. J., *J. Biol. Chem.*, 1939, **131**, 567; Griffith, W. H., *J. Nutrition*, 1940, **19**, 437.

⁴ Channon, A. J., Manifold, M. C., and Eckstein, H. C., *J. Biol. Chem.*, 1940, **135**, 886.

⁵ Tucker, H. P., Trendwell, C. R., and Eckstein, H. C., *J. Biol. Chem.*, 1940, **135**, 85.

⁶ Bloor, W. R., *J. Biol. Chem.*, 1916, **24**, 227.

⁷ Bloor, W. R., and Knudson, A., *J. Biol. Chem.*, 1916, **27**, 107.

⁸ King, E. J., *J. Biol. Chem.*, 1932, **20**, 240.

TABLE II.

Fibrinogenolysis by Varying Strength of Streptokinase Solution.

L = 5 cc H.F. + 0.5 cc buffer + 0.5 cc strep. (strengths cited), incubated at 40°C for periods stated. Clotting-times (sec.) at 25°C for 0.5 cc L + 0.5 cc T (1%).

L.	Strength strep. %	15 sec.	$\frac{1}{4}$ hr	$\frac{1}{2}$	$\frac{3}{4}$	1	2	2½	3	4	8	18 hr
		sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.
1.	0.05	6	7	8	11	13	25	35	45	55	360	∞
2.	0.1	6	9	13	21	37	174	300	∞			
3.	0.5	6	16	46	105	∞						
4.	1.0	6	19	65	186	∞						

TABLE III.

Fibrinogenolytic Tests for Tryptogen in Plasma Fractions.

L = 5 cc cited fractions + 0.5 cc strep. (1%) incubated at 40°C for periods stated. Clotting times (sec.) for 0.5 cc L + 0.5 cc T (1%).

L.	Fraction	15 sec	$\frac{1}{4}$ hr	$\frac{1}{2}$	$\frac{3}{4}$	1	1¼	1½	2	5	21 hr
		sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.
1.	H.F.	7	9	10	55	420	∞				
2.	B.F.	5	5	5	5	5	5	5	5	5	5
3.	H.F. B.F.	5	5	5	9	25	100	∞			
4.	H.Fb.	6	24	∞							
5.	B.Fb.	5	5	5	5	5	5	5	5	5	5
6.	B.Fb. H.F.	5	5	5	10	45	260	∞			

terials, on the other hand, are remarkably stable. B.F. remains clear and gives identical c.t., with T, for 7-10 days at room temperature. The fibrin clots show no visible change in 10-14 days at 40°C.

Fibrinogenolysis by varying strengths of streptokinase solution. (Table II). The clear supernatant from extracting 100 mg crude streptokinase with 10 cc buffer solution is decanted and filtered through glass-wool to yield a solution designated "1%," from which dilutions are made for the tests of Table II. Concentrations of 0.5-1.0% are about optimal and weaker solutions give slower fibrinogenolysis in H.F. The streptokinase extraction for use in these tests requires fresh preparations, since solutions rapidly lose potency and are inactive within 24-48 hours even at ice-box temperature. Solutions of 0.01% (or less) show no fibrinogenolysis but continue to give the same (6'') c.t. as controls (buffer only) in tests extending over 18 hours at 40°C.

Fibrinogenolytic tests for tryptogen in plasma fractions. (Table III). Recent work-

ers¹⁷⁻¹⁹ have shown that streptokinase is not, as long believed,¹³ itself the proteolytic agent, but merely the kinase or activator of enzyme-precursor (tryptogen) in plasma. The extent to which one-tenth volume of 1% streptokinase can activate the tryptogen in the present plasma fractions is brought out by the fibrinogenolytic tests of Table III. The high content of tryptogen in H.F. and H.Fb. is clearly shown as compared with the absence of tryptase development in B.F. and B.Fb. That the failure to lyse B.F. and B.Fb. is due to lack of enzyme precursor rather than to presence of inhibitors in the bovine material is shown in Exp. 3 (and 6), where a mixture of H.F. and B.F. (or B.Fb.) shows complete lysis of *all* the fibrinogen, bovine as well as human, in very little longer time than the human alone (L₁). These data also rule out any strict species specificity and some similar experiments on dog fibrinogen add further evidence on this point.

¹⁸ Kaplan, M. H., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 40.

¹⁹ Christensen, L. R., and MacLeod, C. M., *J. Gen. Physiol.*, 1945, **28**, 559.

¹⁷ Milstone, H., *J. Immunol.*, 1941, **42**, 109.

TABLE I.
Clotting-times in Relation to Concentration of Plasma Fraction.*
Clotting-times (sec.) at 25°C for 0.5 cc F (strengths* cited) + 0.5 cc T (1%).

F.	Plasma fraction	Strength* of solution (% dry weight of original fraction):						
		2.0	1.0	0.5	0.2	0.1	0.05	0.02
1.	H.F.	sec. 7	sec. 6	sec. 6	sec. 8	sec. 14	sec. 35	sec. 115
2.	H.Fb.	6	5	5	8	13	34	136
3.	B.F.	6	5	5	6	7	10	47
4.	B.Fb.	7	6	5	6	8	18	126

* Fractions I contain about 60% coagulable fibrinogen, whereas the fibrinogens are about 90% coagulable.

2. H.Fb.: human fibrinogen, purified, courtesy of Harvard workers; 3. B.F.: bovine plasma Fraction I, courtesy of Dr. Lesh (Armour Laboratories);[†] 4. B.Fb.: fibrinogen, repptd. $\times 2$ from B.F. by $\frac{1}{4}$ sat. $(\text{NH}_4)_2\text{SO}_4$; 5. Strep.: streptokinase (miscalled "streptococcal fibrinolysin") prepared according to the methods of Tillett and Garner.¹³ Extraction of 100 mg with 10 cc borate buffer¹ yields a clear supernatant which is removed from the insoluble residue for use in the tests; 6. T: thrombin, enzyme-free, in the form of "rabbit hemostatic globulin," courtesy of Dr. Parfentjev (Lederle Labs).¹⁴ The dried material is soluble in 1.0% solution in borate buffer; 7. Hep.: Sod. heparinate (Lederle), 1 mg = 100 Toronto units, 1% soln.; 8. T.I.: crystalline trypsin-inhibitor, from pancreas, courtesy of Dr. Kunitz (Rockefeller Institute, Princeton);¹⁵ 9. S.B.I.: crystalline trypsin-inhibitor, from soybean, courtesy of Dr. Kunitz;¹⁶ 10. Egg: commercial egg-albumen ("albumin, egg, soluble powder," Merck), a preparation in laboratory stocks for many years; 11. E.I.: a purified inhibitor from egg-white, sent us by Dr. T.

E. Weichselbaum (Washington Univ., St. Louis) 6 years ago; 12. Buffer: borate buffer,¹ pH = 7.7, used as solvent and diluent throughout.

Clotting-time in relation to concentration of plasma fraction (Table I). Percentage concentration is in terms of dry weight of original material. It is not corrected for small amounts of insoluble matter and for somewhat significant contamination with non-coagulable protein. Fractions I yield about 60% true fibrin, whereas fibrinogens are about 90% clottable. The data in Table I vary slightly from experiment to experiment but the values do afford a fair approximation of the amounts of fibrinogen equivalent to the various clotting-times (c.t.) throughout the present studies. Solutions of 0.5-1.0% give optimal clotting-times and are used throughout. Such concentrations are admittedly high for maximal sensitivity of fibrinogenolytic tests but are preferred for testing their content of proteolytic enzyme system.

Fibrinogenolytic tests for tryptase in plasma fractions (H.F., H.Fb., B.F., and B.Fb.) There is no demonstrable fibrinogenolysis in these 4 plasma fractions in 24-48 hr. Clotting tests of 0.5% solutions, sampled at intervals and added to 0.5 cc T, gave c.t. of 5-6 sec. except in H.F. (48 hr.) and B.Fb. (24 hr.) where 10" and 14" c.t., respectively, were, due to removal of some fibrinogen by "spontaneous" clotting, of very minor importance. That minute traces of tryptase are present, nevertheless, in the human fractions is indicated by lysis of their fibrin clots on overnight incubation at 40°C. Bovine ma-

¹¹ Cohn, E. J., et al., *J. Am. Chem. Soc.*, 1946, **68**, 459.

¹² Edsall, J. T., Ferry, R. M., and Armstrong, S. H., Jr., *J. Clin. Invest.*, 1944, **23**, 557.

[†] Lesh prepares bovine plasma fractions on the principles worked out at Harvard.

¹³ Garner, R. L., and Tillett, W. S., *J. Exp. Med.*, 1934, **60**, 239.

¹⁴ Parfentjev, I. A., Goodline, M. A., and Clapp, F. L., *J. Lab. Clin. Med.*, 1943, **28**, 1465.

¹⁵ Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, 1936, **19**, 991.

¹⁶ Kunitz, M., *J. Gen. Physiol.*, 1946, **29**, 149.

TABLE V.
Fibrinogenolytic Tests of Trypsin Inhibitors.

Lytic mixture (L) = 5 cc H.F. (0.5%) or H.Fb. (1%) + 0.5 cc strep. (1%) + 0.5 cc cited inhibitor, incubated at 40°C for periods stated. Clotting-times (sec.) at 25°C for 0.5 cc L + 0.5 cc T (1%).

L.	Fraction	Inhibitor	15 sec	¼ hr	½	¾	1	1¼	1½	2	2¼	2½	3	8	12	24 hr
			sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.
1.	H.F.	none	5	13	27	64	95	320	∞							
2.	H.F.	egg (1%)	5	7	10	13	19	29	37	83	123	∞				
3.	H.F.	S.B.I. (1%)	6	6	6	6	6	6	6	6	6	6	6	6	6	6
4.	H.Fb.	none	6	24	∞											
5.	H.Fb.	E.I. (1%)	5	8	12	16	20	27	35	53	—	—	95	∞		
6.	H.Fb.	T.I. (1%)	6	6	6	6	6	6	6	6	—	—	6	6	6	6

(original 7" clotting-time) in T.I., mere trace (10" c.t.) in S.B.I., and advanced lysis (480" c.t.) in E.I. The last, therefore, acted weakly but the crystalline inhibitors were very effective. T.I. showed no lysis whatever in 48 hours, whereas S.B.I. gave clotting-times 25", 114", 168" at 24, 36 and 48 hours, respectively, showing that the tryptase action finally broke through the inhibition. It is perhaps noteworthy that tryptase activity is demonstrable after such long periods, despite the evidence (Table IV) of a definite degree of instability.

Streptokinase, etc. In the absence of plasma protease (tryptase) or its precursor (tryptogen), the streptokinase lacks proteolytic, thrombic, or thromboplastic effects. It is possible, of course, to obtain tryptase in fibrinogen-free plasma fractions. The very important thromboplastic action of this plasma protease will be reported in a subsequent communication.

Nomenclature. Similarities to pancreatic trypsin, e.g. 1. alkaline pH optimum, 2. variety of proteins attacked (fibrin, fibrinogen, prothrombin, gelatine, casein, hemoglobin, etc.), 3. effects of known trypsin-inhibitors, and 4. clot-aiding actions, favor continued characterization of the plasma protease as a "tryptase" or trypsin-like enzyme.¹ The new term *plasmin*¹⁹ is acceptable but hardly necessary and, pending isolation, perhaps somewhat premature. The ambiguous term "*fibrinolysin*," which has been used both for the tryptokinase²² and for the active tryptase²³ should obviously be avoided.

Summary. The present study is based upon the use of a fibrinogenolytic (rather than fibrinolytic) technic for demonstration of a natural plasma protease (*tryptase*) and its precursor (*tryptogen*) in the fibrinogen-rich plasma fractions. The value of *streptokinase* (miscalled "streptococcal fibrinolysin") for activation of the enzyme precursor is confirmed and tests are presented to show that failure to secure proteolytic effects e.g. in bovine plasma Fraction I, is due to lack of tryptogen (tryptase precursor) rather than to any significant presence of tryptase-inhibitor.

Heparin, unaided, shows no enzyme-inhibitory effects in the cited tests. *Antitryptase* effects of (a) crystalline inhibitors, from pancreas and soybean, and (b) crude and fractionated egg-albumen, favor the characterization of the plasma protease as a "tryptase."

Confirmation of the existence of tryptogen, together with a trace of active tryptase, in H.F. ("antihemophilic globulin") is further, but still inconclusive, evidence that the source of the coagulation defect in hemophilia is to be sought for in the complexities of the plasma protease system.

The human plasma fractions used in this work were prepared from blood collected by the American Red Cross under a contract between the Office of Scientific Research and Development and Harvard University.

²² Kaplan, M. H., *J. Clin. Invest.*, 1946, **25**, 331.

²³ Loomis, E. C., George, C., Jr., and Ryder, A., *Arch. Biochem.*, 1947, **12**, 1.

TABLE IV.

Fibrinogenolytic Tests of Effects of Heparin on Trypsin Formation and Stability.

Lytic mixtures at 40°C for periods stated. All reagents 0.5%. Clotting-times (sec.) at 25°C for 0.5 cc L + 0.5 cc T (1%). L₁ (without heparin): tests 3-6. L₂ (with heparin): tests 7-10.

L.	Lytic mixture	15 sec	¼ hr	½	¾	1	2	4	6	17	24 hr
		sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.
1.	10 cc H.F. + 1 cc buffer + 1 cc Strep.	6	16	46	105	∞					
2.	10 cc H.F. + 1 cc Hep. + 1 cc Strep.	25	±	∞							
3.	5 cc B.F. + 1 cc L ₁ (15' old)	6	6	6	—	6	7	11	20	480	∞
4.	5 " " + 1 " " (25' ")	6	6	6	—	6	6	8½	10	100	165
5.	5 " " + 1 " " (50' ")	6	6	6	—	6	6	7	8	35	55
6.	5 " " + 1 " " (100' ")	6	6	6	—	6	6	6	6	14	16
7.	5 " " + 1 " " L ₂ (15' ")	12	12	13	—	14	18	28	40	180	340
8.	5 " " + 1 " " (25' ")	12	12	12	—	13	16	20	28	140	195
9.	5 " " + 1 " " (50' ")	12	12	12	—	12	14	16	21	52	60
10.	5 " " + 1 " " (100' ")	12	12	12	—	12	13	14	17	34	34

Instability of activated trypsin and effects of heparin. (Table IV). Some preliminary tests showed that 1 cc of (H.F. + strep.) lysate had a stronger effect in lysing 5 cc of fresh H.F. if added at (or before) the time of complete lysis in the original mixture than if added ¼-½ hour later. Because of the finding that heparin apparently enhances the potency of the trypsin, and because this might possibly be due to some stabilization of the enzyme, the stability tests of Table IV were conducted with and without heparin. At incubation periods of 15, 25, 50, 100 minutes, 1 cc samples were removed from (H.F. + strep.) lytic mixtures (a) without (L₁) and (b) with (L₂) one-tenth volume of 0.5% heparin (v. reagents). On addition of the 1 cc samples to 5 cc B.F. (0.5%), and observation of the rather slow fibrinogenolysis, the activity differences are clearly brought out. The instability of trypsin occurs in both series, so there is no evidence that heparin is stabilizing the enzyme. The best explanation of the *apparent* improvement of trypsin activity in the heparinized mixtures is that there is some immediate antithrombic action of heparin.^{20,21} While this merely increases the clotting-time from 6" to 12" while the fibrinogen is at, or close to, full strength, it would become more signifi-

cant as the fibrinogen is weakened by tryptic lysis. In support of this explanation, mixtures of B.F. diluted to contain in 0.5 cc volume 0.5, 0.2, 0.1, 0.05% of protein, and all treated with 0.05 cc of heparin (diluted to give the identical concentration of the above tests) and then clotted with the usual 0.5 cc T, give clotting-times: 12", 18", 35", 100" as compared with controls (without heparin): 5½", 6", 7", 10". Obviously, the antithrombic effect of heparin is much stronger with weaker fibrinogen concentrations.

Fibrinogenolytic tests of trypsin inhibitors. (Table V). The effects of known inhibitors (of pancreatic trypsin) on the natural plasma trypsin system are illustrated by selected tests, using both H.F. and H.Fb., in the first instance, on the original protein + streptokinase mixture. Inhibition is readily revealed by the fibrinogenolytic test method and is demonstrated for (a) crystalline trypsin-inhibitors, both from pancreas (T.I.) and from soybean (S.B.I.), and (b) egg-albumen, crude (Egg.) and fractionated for trypsin-inhibitor (E.I.).

In a second approach, in confirmation of the ability of these inhibitors to inactivate fully-formed trypsin, not merely to prevent its formation (possible antikinase action), additional tests were made on mixtures: 5 cc B.F. + 0.5 cc fresh lysate (H.F. + strep. incub. 35 min.) + 0.5 cc inhibitor solution. The control (buffer only) lysed in 12 hours, at which time there was no lysis

²⁰ Ferguson, J. H., and Glazko, A. J., *Am. J. Physiol.*, 1941, 134, 47.

²¹ Glazko, A. J., and Ferguson, J. H., *Am. J. Physiol.*, 1941, 134, 54.

TABLE V.
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L.	Fraction	Inhibitor	15 sec	¼ hr	½	¾	1	1¼	1½	2	2¼	2½	3	8	12	24 hr
			sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.
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4.	H.Fb.	none	6	24	∞											
5.	H.Fb.	E.I. (1%)	5	8	12	16	20	27	35	53	—	—	95	∞		
6.	H.Fb.	T.I. (1%)	6	6	6	6	6	6	6	6	—	—	6	6	6	6

(original 7" clotting-time) in T.I., mere trace (10" c.t.) in S.B.I., and advanced lysis (480" c.t.) in E.I. The last, therefore, acted weakly but the crystalline inhibitors were very effective. T.I. showed no lysis whatever in 48 hours, whereas S.B.I. gave clotting-times 25", 114", 168" at 24, 36 and 48 hours, respectively, showing that the tryptase action finally broke through the inhibition. It is perhaps noteworthy that tryptase activity is demonstrable after such long periods, despite the evidence (Table IV) of a definite degree of instability.

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²² Kaplan, M. H., *J. Clin. Invest.*, 1946, **25**, 331.

²³ Loomis, E. C., George, C., Jr., and Ryder, A., *Arch. Biochem.*, 1947, **12**, 1.

The Serological Differentiation of Mumps Complement-Fixation Antigens.*

GERTRUDE HENLE, WERNER HENLE, AND SUSANNA HARRIS.

From the Children's Hospital of Philadelphia (Department of Pediatrics, School of Medicine, University of Pennsylvania), Philadelphia, Pa.

The recent adaptation of mumps virus to the chick embryo by Habel¹ and others^{2,3} has greatly facilitated the serological diagnosis of the disease in its various manifestations and has placed such tests within the reach of the routine laboratory. The occurrence of a hemagglutination phenomenon similar to that observed with the influenza viruses permits the determination of antibodies by the inhibition of this reaction, as shown by Levens and Enders.² In addition, various materials derived from the infected chick embryo have been shown to contain complement-fixation antigen. Following the inoculation of the virus by different routes antigen was found in suspensions of the allantoic,² amniotic² and yolk sacs,^{1,3} and in the allantoic¹ and amniotic fluids.^{1,2} Studies in this laboratory, aside from confirming the earlier observations, were concerned with the demonstration of differences in the mumps-specific antigenic properties of suspensions of infected allantoic and amniotic sacs on the one hand, and of allantoic and amniotic fluids on the other.

Methods and Materials. A strain of mumps virus adapted to the amniotic sac of the chick embryo was kindly supplied by Dr. Enders. It has been propagated in this laboratory both in the amniotic and allantoic sacs of 8-day-old chick embryos for 8 to 14 consecutive passages, using for the transfers 10- to 10,000-fold diluted amniotic or allantoic fluids, respectively. For both routes a hole with a diameter of about 1 cm was cut with scissors into the shell over the air

sac after the shell had been sterilized with 70% alcohol. In the case of the allantoic inoculation the needle was slipped just under the membrane to assure injection of the seed into the allantoic sac which is still very small on the 8th day of incubation of the embryo. For the amniotic inoculation a small part of the exposed shell membrane was removed over the approximate location of the embryo and the inoculum was deposited into the amnion under full visibility of this structure. The hole in the shell was sealed with scotch tape. After further incubation of the eggs at 35 to 37°C for 5 to 7 days, the virus usually attained maximal concentration in the respective embryonic fluids as measured by infectivity titrations in the chick embryo, hemagglutination and complement-fixation tests. At this time the allantoic and amniotic fluids and the corresponding membranes were harvested separately. The membranes were emulsified by means of a Waring blender in saline solution to form a 10 or 20% suspension.

Infectivity titrations were performed in 8-day-old chick embryos by the injection of the virus preparation diluted in steps of 10 in broth by the allantoic (0.5 ml inoculum) or the amniotic routes (0.2 ml inoculum) depending on the passage series of virus used.

For the hemagglutination test 0.4 ml of virus suspension was mixed with 0.2 ml of a 1% suspension of washed fresh chicken red cells and the mixtures were incubated at 4°C until the erythrocytes had settled. The degree of agglutination was determined by the pattern formed by the cells at the bottom of the test tubes.

The complement-fixation technic was the same as employed for the studies on the antigenic differentiation of influenza antigens.⁴ Dilutions of antigen, serum and

* The work described in this paper was aided by the Office of Naval Research.

¹ Habel, K., *Pub. Health Rep.*, 1945, **60**, 201.

² Levens, J. H., and Enders, J. F., *Science*, 1945, **102**, 117.

³ Beveridge, W. I. B., Lind, P. E., and Anderson, S. G., *Austral. J. Exp. Biol. and Med. Sci.*, 1946, **24**, 15.

⁴ Wiener, M., Henle, W., and Henle, G., *J. Exp. Med.*, 1946, **83**, 259.

guinea pig complement were mixed using 0.1 ml amounts of each. The mixtures were incubated at 37°C for one hour before the addition of 0.2 ml of sensitized sheep red cells (2.5% suspension). After further incubation for 20 minutes at 37°C and overnight at 4°C, the tests were read by estimating the degree of hemolysis obtained. Two units of amboceptor and 1.3 units of "Lyovac" complement[†] were employed. The highest initial dilutions of antigen or serum giving complete fixation of complement (no hemolysis) were recorded as titers. Most tests were performed according to optimal titration technique since zone phenomena were observed frequently in agreement with earlier observations by Enders and co-workers.⁵ Human convalescent sera were used in these studies and the results were compared whenever possible with those obtained with sera from the same patients taken during the first days of the disease. Control antigens were prepared from suspensions of allantoic and amniotic membranes derived from normal embryos, or from eggs infected with influenza B virus, in a manner corresponding to that used for the mumps preparations.

Experimental. Upon allantoic inoculation of mumps virus of the allantoic-passage series, complement-fixation antigen was found in the allantoic fluids and membranes and in the amniotic sacs, but none or little in the amniotic fluids. The allantoic tissue usually contained higher titers of antigen than the amniotic sac. Conversely, following amniotic injection of virus of the amniotic-passage series, higher titers of antigen were noted in the amniotic sac than in the allantoic tissue and, in early passages, the allantoic fluid gave negative or weak reactions in contrast to the strongly positive results with the amniotic fluids. Normal allantoic and amniotic fluids did not react with any of the sera used, whereas positive reactions with the suspensions of normal allantoic and amniotic membranes were obtained with some of the sera in low dilution. The antigen

titers in the infected fluids, varying between 1:2 and 1:8, were lower than those observed in the corresponding membranes which were found to lie between 1:16 and 1:128. Hemagglutination tests, on the other hand, showed high titers in the fluids (1:256 to 1:1024 for allantoic, and 1:1024 to 1:8192 for amniotic fluids) but were low or negative with the suspensions of membrane. Although interpretation of the results of hemagglutination tests with the tissue suspensions has to take into consideration the inhibitory effect of some component in the emulsion, the data are comparable to those of infectivity titrations. In these the fluids revealed titers 100 to 1000 times higher than the suspensions of the membranes. The number of 50%-infectivity doses (ID₅₀) in the fluids varied from 10^{7.3} to 10^{8.8} per ml in the reported experiments, whereas the suspensions of membranes gave values between 10^{4.5} and 10^{7.8}. These observations failed to show a correlation between the infectivity and hemagglutinin titers on the one hand, and the concentration of complement-fixation antigen on the other. This discrepancy has already been noted by Levens and Enders.²

These data suggested further comparisons among the various materials in regard to the nature of the antigens they contained. By the use of high speed centrifugation it could be shown that the mumps antigens of allantoic and amniotic fluids were sedimented together with the virus particle at 20,000 r.p.m. for 20 minutes. The infective, hemagglutinating and complement-fixing properties were found in the sediment whereas the supernatant fluid gave negative complement-fixation tests and only low titers of virus and hemagglutinin. This is in agreement with the findings of Beveridge and Lind.⁶ From the tissue suspensions, on the other hand, only part of the complement-fixation antigen was removed by this procedure although the infectivity of the supernatant fluid showed a decrease in titer comparable to that observed with infected allantoic and amniotic fluids. The hemagglutination re-

[†] Generously supplied by Sharp and Dohme, Inc.

⁵ Enders, J. F., Cohen, S., and Kane, W. W., *J. Exp. Med.*, 1945, **81**, 119.

⁶ Beveridge, W. I. B., and Lind, P. E., *Austral. J. Exp. Biol. and Med. Sci.*, 1946, **24**, 127.

TABLE I.
Differential Centrifugation of Allantoic Fluid and Membrane Suspension Infected with Mumps Virus.

Preparation	Allantoic fluid			Allantoic membrane		
	Infectivity (allantoic inoculation), ID ₅₀ /ml	Hemagglu- tination titer	Complement- fixation maximal titer	Infectivity (allantoic inoculation), ID ₅₀ /ml	Hemagglu- tination titer	Complement- fixation maximal titer
Original	107.5	1:1536*	1:8†	105.5	0	1:64
20,000 r.p.m. supernate	105.3	1:8	0	105.5	0	1:32
20,000 r.p.m. sediment 2 × conc.	108.3	1:4096	1:16	105.3	1:32	1:16
30,000 r.p.m. supernate	103.1	0	0	102.8	0	1:4
30,000 r.p.m. sediment 2 × conc.	103.3	0	0	101.8	0	1:32

* Initial dilution of the preparation giving last definite agglutination of red cells.

† Initial dilution of serum or antigen giving last complete fixation of complement.

All serum and antigen controls, as well as controls with acute serum and with normal membrane preparation gave negative results.

action, which usually was negative in the original suspensions, became distinctly positive in the resuspended sediments. This finding may be explained by the assumption that the hemagglutinins have been separated by the centrifugation procedure from an inhibitor present in the original suspension.† Recentrifugation of the 20,000 r.p.m.-supernate at 30,000 r.p.m. for one hour removed a large part of the "soluble" antigen, but some of it still remained in suspension. An experiment of this type, employing infected allantoic fluids and the corresponding membranes as the source of antigen, is shown in Table I. As pointed out, control antigens prepared from normal allantoic and amniotic sacs gave positive reactions with a few of the sera in low dilution. The material showing this reactivity was in part sedimented at 20,000 r.p.m., the supernatant being usually inert. It is obvious then that tissue antigens for diagnostic tests should be subjected to high speed centrifugation in order to avoid some of the nonspecific reactions.

The centrifugation experiments showed that physical differences existed between the antigen present in the embryonic fluids and at least part of the antigen present in suspensions of the membranes. In further experiments comparisons were made between preparations of infected allantoic fluid on the one hand, and the supernatant fluid after centrifugation at 20,000 r.p.m. of suspended allantoic membrane on the other; the former representing mostly large antigen particles which are sedimentable with the virus activity, the latter containing a smaller antigen not directly linked with the infective property. These comparisons showed on occasion differences in the optimal titration patterns of the 2 antigens but not as frequently and as markedly as was noted in the case of the corresponding influenza antigens.^{4,7} More convincing evidence of serological differentiation was obtained by absorption of convalescent sera with the 2 types of

‡ Similar results were obtained upon dialysis of suspensions of infected membranes.

† Henle, W., and Wiener, M., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 176.

TABLE II.
Results of Serum Absorption Tests.

Serum absorption		Optimal complement-fixation reaction Antigen									
		Allantoic fluid			Allantoic membrane						
Material	1D ₅₀ /ml serum	Units of antigen/ml serum	Dilution of serum	Orig. und.	20,000 R.P.M. sediment und.	Orig. 1:16	Supernate 1:16	Sediment 1:2	Supernate 1:4	Sediment 1:8	Saline
—	—	—	1:8 1:16 1:32 1:64	0 0 0 tr	0 0 0 tr	0 0 0 wk	0 0 0 wk	0 0 0 0	0 0 0 st	0 0 0 tr	r e e r
Allantoic fluid 20,000 R.P.M. sediment	109.1	320	1:8 1:16 1:32 1:64	st e e e	st e e e	0 0 ac e	0 0 ac e	0 0 ac e	0 wk e e	0 0 ac r	r e e r
Allantoic membrane 20,000 R.P.M. sediment	105.9	1280	1:8 1:16 1:32 1:64	0 0 ac e	0 0 tr e	0 e e e	0 e e e	0 e e e	0 e e e	0 e e e	e e e r
Allantoic membrane 30,000 R.P.M. sediment	102.2	320	1:8 1:16 1:32 1:64	0 0 0 ac	0 0 0 tr	0 e e e	0 e e e	st e e e	0 e e e	0 e e e	r e e e

0—no hemolysis; tr—trace; wk—weak; st—strong; ac—almost complete; e—complete hemolysis.

TABLE III.
Antibody Content of Various Selected Human Sera.

Case No.	Age (years)	History	Time after onset of disease	Serum antibody titer against		
				Virus-bound antigen (allantoic fluid)	Soluble antigen (Supernate 20,000 R.P.M. of allantoic membrane)	Normal allantoic membrane
1	4½	mumps	3 days	1:4	1:16	1:2
			15 "	1:64	1:64	1:2
			29 "	1:128	1:128	1:2
			46 "	1:128	1:64	1:2
2	5½	"	7 "	1:16	1:16	0
3	4	"	7 "	1:16	1:32	0
			17 "	1:64	1:64	0
4	6½	"	4 "	1:16	1:8	0
			14 "	1:64	1:32	0
6	4	"	4 "	1:4	1:8	<1:4
			16 "	1:32	1:64	<1:4
36	9	"	2 months	1:64	1:8	0
35	7	"	3 "	1:32	1:8	0
31	8	"	2 years	1:8	0	0
28	2	"	2 "	1:8	0	0
11	4	"	2 "	1:4	0	0
25	10	"	2 "	1:8	0	0
13	11	"	2 "	1:4	0	0
44	9	unknown	—	1:16	0	0
43	6	"	—	1:8	0	0
42	9	"	—	1:8	0	0
19	8	inapparent mumps	16 days†	1:128	1:32	0
16	6	"	39 "†	1:16	1:4	0

* Partial fixation of complement in serum dilution 1:4.

† Negative serum obtained 16 and 39 days, respectively, prior to the recorded specimen.

antigen. Human convalescent serum was absorbed with the sediments obtained by centrifugation at 20,000 r.p.m. from infected allantoic fluid or suspension of allantoic membrane, or with the material sedimented at 30,000 r.p.m. from the suspension of membrane after removal of the larger particles at 20,000 r.p.m. The ID₅₀ for chick embryos as well as the units of antigen used for the absorption per 1 ml of serum were determined and are recorded in Table II. When mumps convalescent serum was absorbed with the antigen sedimented at 20,000 r.p.m. from allantoic fluid (virus particles) it no longer reacted with this antigen nor with the original allantoic fluid. It gave, however, distinctly positive reactions with suspensions of infected membrane and the fractions derived therefrom, although a decrease in serum titer was noted. On the other hand, absorption of the serum with the smaller antigen sedimented at 30,000 r.p.m. from suspensions of allantoic membrane previously cleared by centrifugation at 20,000

r.p.m. removed the reactivity with the "soluble antigen" but left antibodies to the allantoic fluid antigen and the sediment derived therefrom. Absorption of the serum with the particles sedimented from the membrane suspension at 20,000 r.p.m. gave results corresponding to the effect of absorption with the soluble antigen rather than with the virus particles obtained from allantoic fluid. The reasons for this latter finding are not clear as yet. The infectivity titrations of the suspensions of membrane used for the absorption showed only 0.1% of the activity of the allantoic fluid material. Consequently, there might not have been a sufficient concentration of virus to remove the antibodies to the virus-bound antigen. On the other hand, antibodies against the soluble antigen were completely removed by the particles sedimented from the membranes at 20,000 r.p.m. It is possible then that the "soluble antigen" occurs in different physical states, i.e., in different particle sizes either *per se*, or as a result of adsorption onto par-

ticulate components of the host cell. One also might consider the possibility that the virus may occur in a different phase in the tissues, *i.e.*, covered with the soluble antigen, as compared to the "naked" virus in the allantoic fluid. This problem requires further investigation. Control absorptions of the convalescent serum with fractions sedimentable at 20,000 and at 30,000 r.p.m. from normal allantoic membranes or from tissues infected with the Lee strain of influenza B virus did not affect the reactivity of the serum with either the mumps virus as contained in the allantoic fluid or the mumps soluble antigen present in the suspension of membrane.

Further evidence of the serological differentiation between the 2 antigens was obtained in the study of the reactivities of various human sera with these preparations. Marked titers in antibodies against both antigens were found in sera of children convalescent from mumps and those having undergone a recent inapparent infection. Some sera from individuals with a history of long past infection or from subjects with unknown exposure showed antibodies only against the virus-bound antigen but not against the soluble material. Some of these cases are listed in Table III. This finding emphasizes again the difference between the 2 antigens; the one dominantly present in the embryonic fluids, the other in the suspensions of infected tissues. These results suggest, in addition, that the antibodies against the virus-bound antigen persist for a longer period of time following the infection than antibodies against the soluble substance. This, however, can be verified only by following the concentration of the 2 antibodies in individual patients over a prolonged period of time. In a few cases it was noted, too, that the antibodies to the soluble material may be-

come measurable and reach higher concentrations earlier than the antibodies to the virus-bound antigen.

The demonstration of 2 distinct complement-fixing antibodies, and particularly the fact that one of them, the antibody against the soluble substance, may be absent from certain sera tends to explain discrepancies in the correlation of the results of complement-fixation tests with the susceptibility of an individual. If only tissue extracts are employed as antigen which contain mainly the soluble material a negative test may be recorded in a subject with a history of mumps at some time in the past. The use of the virus-bound antigen may properly classify such individuals. This latter antigen will also demonstrate past contact with the virus even though the history may not reveal past apparent infection.

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			15 "	1:64	1:64	1:2
			29 "	1:128	1:128	1:2
			46 "	1:128	1:64	1:2
2	5½	"	7 "	1:16	1:16	0
3	4	"	7 "	1:16	1:32	0
			17 "	1:64	1:64	0
4	6½	"	4 "	1:16	1:8	0
			14 "	1:64	1:32	0
6	4	"	4 "	1:4	1:8	<1:4
			16 "	1:32	1:64	<1:4
36	9	"	2 months	1:64	1:8	0
35	7	"	3 "	1:32	1:8	0
31	8	"	2 years	1:8	0	0
28	2	"	2 "	1:8	0	0
11	4	"	2 "	1:4	0	0
25	10	"	2 "	1:8	0	0
13	11	"	2 "	1:4	0	0
44	9	unknown	—	1:16	0	0
43	6	"	—	1:8	0	0
42	9	"	—	1:8	0	0
19	8	inapparent mumps	16 days†	1:128	1:32	0
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ticulate components of the host cell. One also might consider the possibility that the virus may occur in a different phase in the tissues, *i.e.*, covered with the soluble antigen, as compared to the "naked" virus in the allantoic fluid. This problem requires further investigation. Control absorptions of the convalescent serum with fractions sedimentable at 20,000 and at 30,000 r.p.m. from normal allantoic membranes or from tissues infected with the Lee strain of influenza B virus did not affect the reactivity of the serum with either the mumps virus as contained in the allantoic fluid or the mumps soluble antigen present in the suspension of membrane.

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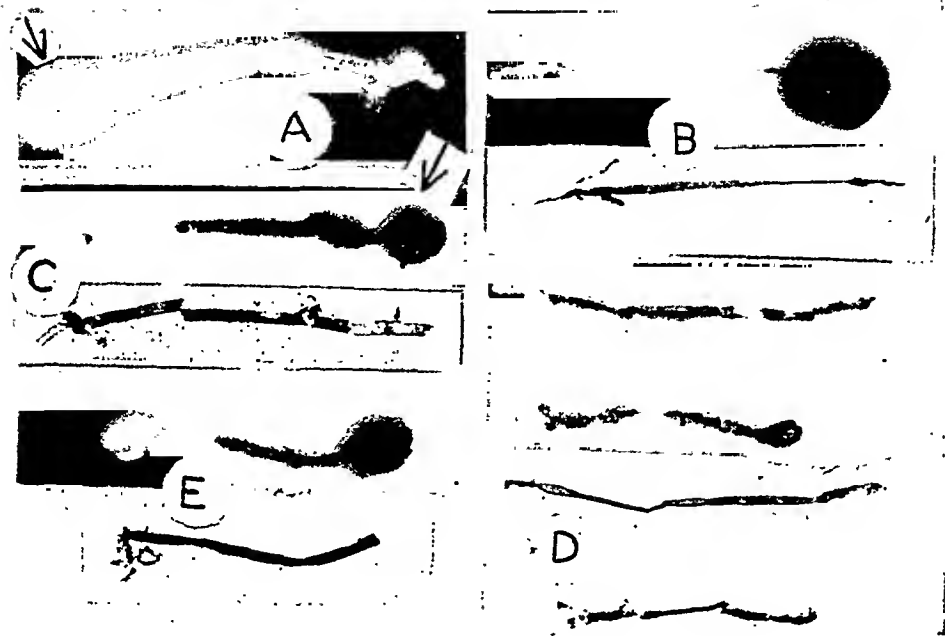


FIG. 1.
Showing the distribution of radiophosphate in *Nitella* and *Chara*. (See text.)

exchange of P^* into such crystals, although the zones of concentration do not correspond with the visible areas of incrustation. (See transmitted light photo).

In photos "E" and "B" are shown radioautographs taken after 60 and 90 minutes of immersion of cells to a depth of 2 mm. Both show concentration of P^* in the node, and some slight localization of P^* along the axis. Both cells are *Nitella*. Photo "D" is a radioautograph of 2 *Nitella* cells together with transmitted light photographs taken after 660 min. immersion in P^* solution to a depth of 2 mm as measured along the cell axis from the arrow. Here the P^* has been distributed throughout the cell, but in discrete patches. One possible interpretation of such distribution is that in the course of vacuum drying of the cells, the protoplasm aggregated in clumps. This does not

seem likely inasmuch as the transmitted light photos show that the chloroplasts must be rather evenly distributed throughout the cell as it is rather uniformly opaque. Microscopic observation confirms such a view. There is also the possibility that in the drying of the cell P^* which has diffused throughout either or both cell wall and protoplasm crystallizes in quite large crystals. The distribution of P^* in these photos, however, seems too sparse to render such a view likely. It would seem that P^* in these algal cells is found in areas where there was already a high concentration of inactive PO_4 for exchange, and that the pictures obtained in this study represent the way P^* is distributed as it diffuses along the cell.

My thanks are due to Professor S. C. Brooks for the use of his laboratory and for many helpful suggestions.

The Localization of Radiophosphate in Cells.

LORIN J. MULLINS.*

From the Department of Zoology, University of California, Berkeley.

The exact mode in which ions are distributed in protoplasm is at present unknown. Using radioactive isotopes, several authors have attempted, by placing photographic film in close contact with tissues and thus obtaining a radio-autograph, to record where added ions are localized in the cell. Unfortunately this procedure has not yielded any great amount of information on cellular localization, although excellent differentiation of different tissue masses has been obtained.¹ The difficulty in obtaining clear radio-autographs of cells is that the cell diameters are of the same order of magnitude as the distance that β -rays, not normal to the film, travel before striking the film. The net result is a general fog which obscures the desired detail. To obtain pictures with more detail it is necessary therefore either to collimate or to focus the electrons coming from the tissue to the film, or else to use cells of much larger dimensions. In this study the second alternative has been employed in that cells of *Nitella* or *Chara* sp., which have diameters of the order of 1-2 mm and lengths of about 40 mm, were used.

Experimental. Cells of *Chara* or *Nitella* were prepared for use as previously described.² The cells were removed from pond water, blotted to remove excess water and placed on end in a tube containing 0.001M radioactive sodium phosphate solution at 15°C and pH 7. In some experiments only enough phosphate solution was used to cover 1-2 mm of the cell as measured along its axis. The tube was then filled with washed paraffin

oil. By this arrangement it was possible to expose only a minimal part of the surface of the cell to phosphate solution and hence obtain some idea of the rapidity of diffusion and possible circulation of the phosphate due to cyclosis. To be sure there must be a thin film of water extending up from the PO_4 solution along the cell surface but this method probably lets ion diffusion proceed as asymmetrically as possible. After various times of immersion the cells were removed from the tubes, washed thoroughly in running distilled water, placed on a glass slide and rapidly frozen and dried *in vacuo*. A piece of X-ray film was then placed over the dried cell and exposed for a suitable time.

Results. Some photographs resulting from these experiments are shown in Fig. 1. In photo "A" the cell (*Nitella*) was immersed in radioactive phosphate solution (P^*) from its end marked by an arrow (\downarrow) for a distance of 25 mm along its axis for a period of 25 minutes. As is to be expected, the P^* concentration is highest in this region, but it can also be seen that the P^* concentration at the node of the cell is quite high. This may be due to the greater mass of protoplasm here or to the more rapid metabolic turnover in this region, or to a combination of both. In photo "C" a cell which was immersed in P^* solution for 25 min. to a depth of only 2 mm is shown. Directly below the radio-autograph (black background) is shown a photo of the cell taken by transmitted light. (The cell was fractured in placing photographic film upon it). It can be seen from this photo that although there is a slight amount of activity in the nodal region, the P^* is mainly concentrated in rather discrete zones along the cell. This cell was *Chara*. The *Chara* cell generally has a rather heavy incrustation of salt deposits on its wall. It is possible that these localized zones of P^* concentration represent the

* Fellow of the American-Scandinavian Foundation at the University Institute for Theoretical Physics, Copenhagen.

¹ Eklundh-Ehrenberg, C., Euler, H. v., and Hevesy, G., *Arkiv f. Kemi.*, 1946, **23A**, 10.

² Brooks, S. C., *Trans. Faraday Soc.*, 1937 **33**, 1002; *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 856.

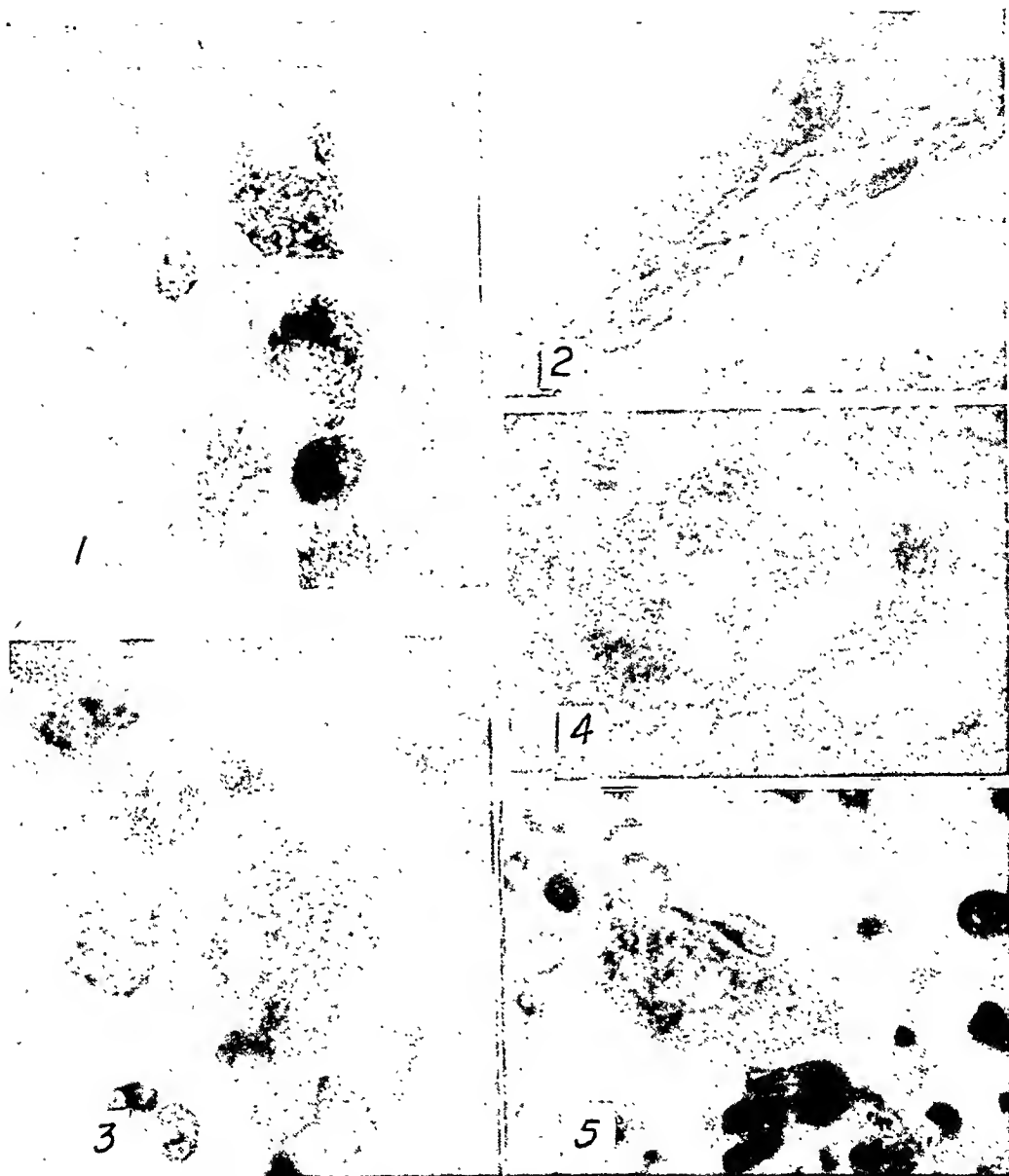


FIG. 1.—Testis of a guinea pig inoculated locally with *Br. melitensis*. Parasitized macrophages. No extracellular brucellae are detected. Formalinized Giemsa. $\times 1000$.

FIG. 2.—Fibroblasts parasitized with *Br. abortus*. Guinea pig testis. Same stain. $\times 900$.

FIG. 3.—Testis of guinea pig. Interstitial cell heavily parasitized with *Br. abortus*. Nyka's stain. $\times 1000$.

FIG. 4.—Same testis. Endothelial cells of a capillary stuffed with *Br. abortus*. Formalinized Giemsa. $\times 1000$.

FIG. 5.—Lung of a mouse inoculated intranasally with *Br. melitensis*. Alveolar cell packed with brucellae. Nyka's stain. $\times 1600$.

strain. After being inoculated, the animals were killed at various intervals in order to follow the infecting organism through the various stages of the process. Smears and

Studies on the Pathogenesis of Brucellosis.*

M. RUIZ-CASTANEDA.

From the Department of Medical Research, General Hospital, Mexico, D.F.

The mechanism by which *Brucella* produces infection and continues to grow in the tissues of immune individuals has been suspected but not clearly demonstrated. One of the most interesting features of the disease in man is the prolonged sequence of periods of illness and apparent recovery for which it has been given the name of undulant fever. The finding of *Brucella* for months and even years after the beginning of the infection places this disease in a class with tuberculosis and syphilis, in which the infecting organism continues to multiply in spite of the considerable degree of immunity displayed by the host. However, brucellae are not quite resistant to cellular and humoral defences as is the case in tuberculosis. Therefore, its ability to remain active within the organs and eventually to be found in the blood stream must depend on factors peculiar to the microorganism.

One of the most interesting observations on the relationship of *Brucella* to the infected tissues was made early in the history of the disease, when Theobald Smith demonstrated that a considerable number of *Brucella abortus* crowded the cytoplasm of the cells of the chorion in the placenta of aborting animals. This finding did not seem to have been fully appreciated as a possible guide in the explanation of the remarkable persistence of *Brucella* in the organism in spite of the coexistence of high degree of immunity.

Corroborating Smith's findings, Goodpasture and co-workers¹ in a study of bacterial

* Read at the First Interamerican Brucellosis Meeting, Mexico, D.F., October 28, 1946.

The expenses of this study were in part defrayed by a grant from Eli Lilly and Co., Indianapolis, Indiana.

¹ Goodpasture, E. W., and Anderson, K., *Am. J. Pathology*, 1937, 13, 149.

² Buddingh, G. J., and Womack, F. C., *J. Exp. Medicine*, 1941, 74, 213.

infection in the developing chick embryo found that *Brucella* multiplied within the cytoplasm of certain cells more intensely than could be observed in extracellular positions. More recently Buddingh and Womack² following the method of Goodpasture found that *Br. abortus* and *suís* had a tendency to select cells of mesodermal origin while the *melitensis* variety was only found in ectodermal cells. The 3 brucellae could be found in considerable numbers within macrophages not merely phagocytized but actually multiplying within the cytoplasm of such cells. These authors advanced the idea that the monocytes acting as a natural means of defense against the infecting agent supplied a favorable refuge where multiplication took place and therefore, constituted the source of continual infection from cell to cell. The intracellular position of *Brucella* in tissues of infected persons was first reported by Meyer³ in a case of acute infection with *Br. suis*. The brucellae were easily seen within parenchyma cells of the kidney. After Meyer's report we decided to investigate intracellular brucellae in fatal cases of human brucellosis. It was discouraging to find so few visible organisms while cultures from the organs showed considerable numbers of colonies. Therefore, it was considered of interest to become familiar with the microscopic aspect of the disease in experimental animals. The following is a presentation of some findings after inoculation of guinea pigs, rabbits and mice with the 3 varieties of *Brucella*. Guinea pigs and rabbits were inoculated intravenously and also directly into the testicle. Mice were inoculated by intravenous and intranasal routes. The strains used as inoculum were *Br. abortus* and *suís* sent to us by Dr. Huddleson and a recently isolated

³ Meyer, K. F., *Essays in Biology*, University of California Press, 1943, pp. 439-459.

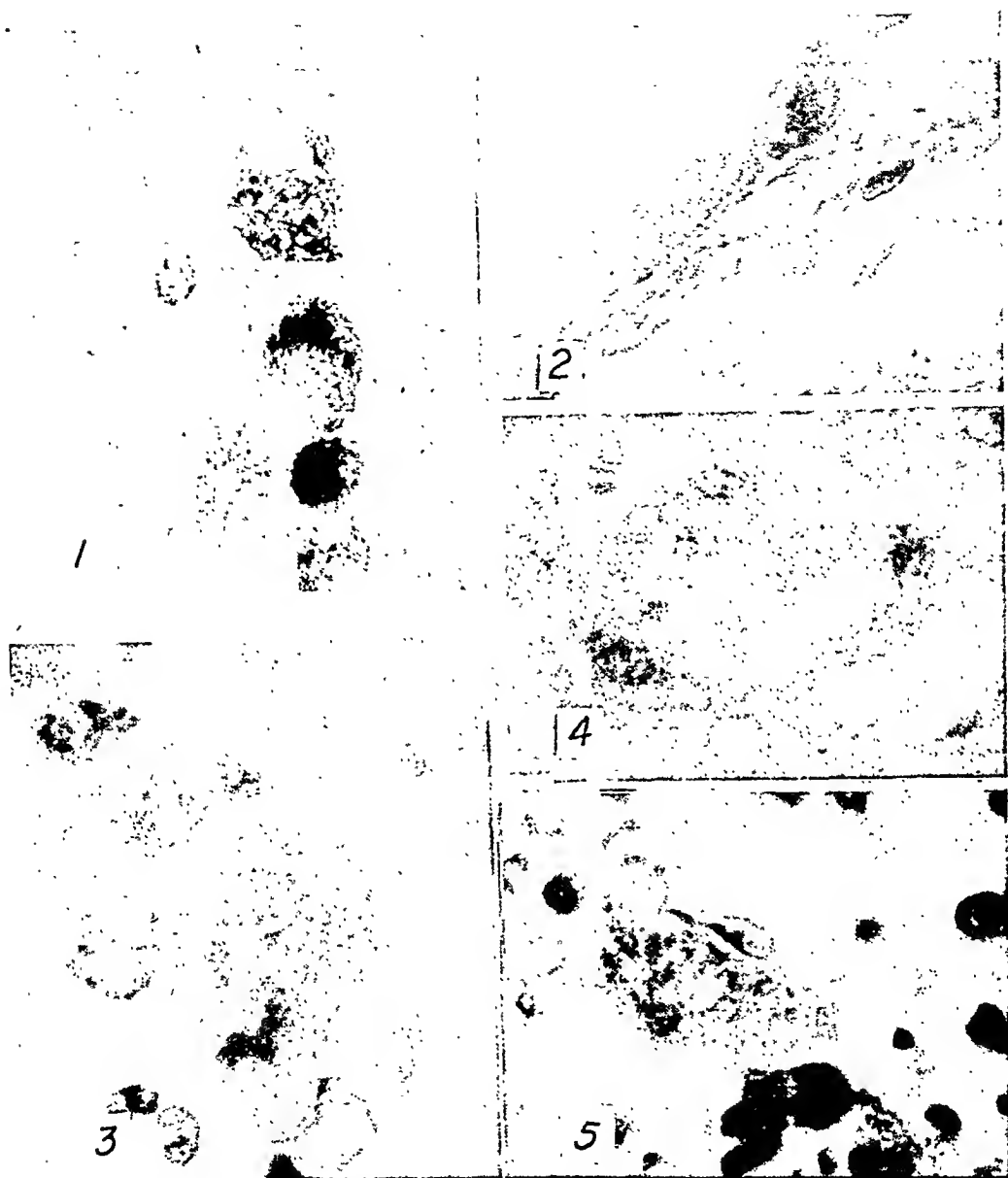


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strain. After being inoculated, the animals were killed at various intervals in order to follow the infecting organism through the various stages of the process. Smears and

prints made from the organs were stained with our methylene-blue-safranin stain for rickettsiae. The sections were stained with formalinized Giemsa or by Nyka's stain.⁴ The latter producing delicately stained cellular elements in which violet stained brucellae could be easily found.

It is unnecessary to include here the observations concerning the cytological response to the infecting organism which will be reported by one of our associates.

The search of brucellae in smears or prints from animals inoculated intracardially showed relatively few extracellular organisms in the spleen and liver, and even less in other organs. Often one could detect polymorphonuclear leucocytes containing few phagocytized organisms and also macrophages containing brucellae in the cytoplasm. The optimum time for the finding of parasitized macrophages was about the 5th day after inoculation. Some of the mononuclear cells contained rather large numbers of brucellae but the microscopic aspect of the parasitized cells was far from being suggestive of intracellular multiplication as reported by Buddingh and Womack. On the other hand, the observation of smears from the testicle of animals inoculated locally showed cells with the cytoplasm crowded with brucellae. In smears from the locally infected testicle there were found a considerable number of polymorphonuclear leucocytes phagocytizing brucellae and also many macrophages stuffed with organisms. Although in sections of the liver and spleen it was not difficult to find infected cells, the testicle afforded better material for the study. Fig. 1 shows cells from the testicle of a guinea pig infected with *Br. melitensis* which leave little doubt of the intracellular growth in cells which are likely to be macrophages. Fig. 2 shows the growth of *Br. abortus* in the cytoplasm of a fibroblast and in Fig. 3 there is no doubt about the multiplication of *Br. abortus* within a cell, probably an interstitial cell, if compared with neighboring cells of similar morphology. In Fig. 4 it seems that cells from a capillary

wall have been parasitized by *Br. abortus*. Most of these preparations were made from animals killed from the 3rd to the 5th day, and it has been our impression that the parasites within the cells have increased not because of phagocytosis but by the actual development of *Brucella* colonies after some of the organisms gained entrance into the cytoplasm. It is interesting to find so few or even no extracellular organisms close to the parasitized cells. However, in certain portions of the testicle one could see large numbers of extracellular organisms and it was usually in zones in which considerable numbers of polymorphonuclear leucocytes were present.

We have not yet sufficient information concerning the type of cells which allow brucellae to develop in the cytoplasm, nor the possible selectivity of the 3 varieties of organisms for a special group of cells. We can only indicate that we found macrophages, fibroblasts and, particularly in the spleen, endothelial and reticular cells which contained brucellae in the cytoplasm. To this we may add parenchymatous cells from the kidney and interstitial cells of the testis.

The inoculation of mice by intravenous or intranasal route has afforded a practical method for the study of the relationship of brucellae to the cells of the infected animal. Even 48 hours after the inoculation it has been possible to find parasitized cells in various organs. In the lung the alveolar cells are found to be parasitized in a similar manner to that which has been observed in typhus. Fig. 5 shows the aspect of an infected alveolar cell which does not look unlike similar cells parasitized with typhus rickettsiae. We have also seen macrophages stuffed with brucellae and polymorphonuclear leucocytes containing phagocytized organisms, usually in small numbers. It is interesting to find that, contrary to what has been observed in typhus-infected animals the bronchial epithelium seems to have no tendency to favor multiplication of brucellae.

Comment. There seems no doubt that most workers experienced in the clinical aspect of brucellosis consider that some of the

⁴ Nyka, W., *J. Pathology and Bacteriology*, 1945, 57, 317.

symptoms shown by the patients are due to a state of hypersensitivity to brucellar antigens. Since 1938 we have advocated this view based on the observation of typical manifestations of allergy and the favorable effect of desensitization of the patients. To illustrate our discussion we shall give a brief description of the general aspect of the caprine type of brucellosis. The disease most frequently begins like typhoid or typhus infection or less frequently as a mild febrile influenza-like infection. The first attack, of variable duration, represents the cyclical phase of the infection ending when the individual has developed sufficient immunity to prevent further injury by the infecting agent. Then, a state of convalescence closes the incident. But in a great number of cases *Brucella* continues to grow as indicated. The cell to cell infection as suggested by Buddingh and Womack insures the preservation of the organism in spite of the state of immunity of the patient. The constant discharge of brucellar products into the system increases this state of immunity and at a certain moment the reactivity of the tissues and the general reactions acquire sufficient intensity to produce considerable discomfort and fever. This constant irritation may become very harmful, overcome the body defenses and even allow considerable multiplication of brucellae either focally or again as a general infection. An important factor in the failure to destroy *Brucella* in the immune individual seems to be the reduction in numbers of the polymorphonuclear leucocytes.

The demonstration of brucellae within cells of various types is a clear indication of the mechanism of preservation of the organisms in the immune individual but most important, for the understanding of the pathogenesis of the numerous clinical manifestations of the infection, seems to be the facility with which *Brucella* are phagocytized by macrophages which may be transported to all parts of the body and become the source of new colonies since the cytoplasm supplies suitable material for the multiplication of the organisms.

Up to the present time we have studied relatively few animals from which only a

limited number of preparations have been suitable for the investigation of intracellular brucellae, therefore, we have not been able to gather sufficient information concerning special affinity of the 3 varieties of the organism for certain cells as observed in the chick embryo by Buddingh and Womack.

According to the observations made by various authors, in experimental brucellosis and in human material, the histological lesions are, fundamentally, the nodules described by Fabyan. The nodular formation could be explained as a reaction around wandering macrophages carrying brucellae or local parasitized cells, which are surrounded first by monocytes and perhaps polymorphonuclear leucocytes and walled off by lymphocytes. It is obvious that in brucellosis there is a considerable call for the latter cells and that the infected individual endeavors to supply them. It seems likely that this mechanism of focal growth of *Brucella* followed by the process of nodule formation explains many of the complications of brucellosis including nervous manifestations, abscesses, focal necrosis, etc. The tendency of brucellae to select the spleen as a refuge during the course of the disease is probably due to exceedingly rich material suitable for the intracellular growth and the hyperplasia observed in this organ must be accounted for the manifestations of hypersplenism often observed in the patients.

Conclusions. The studies referred to, and our own observations in experimental brucellosis are in our opinion, good evidence of the ability of *Brucella* to use the cytoplasm of a variety of cells as a source of material for its growth. The fact that even in the early stages of the infection, when the host has not developed immunity against brucellae, important groups of this organism are found only within the cytoplasm of certain cells, seems to indicate that the intracellular growth is the most convenient for the preservation of the infecting agent. When the intracellular colony has reached its maximum, the cells are destroyed and the brucellae exposed to unfavorable conditions, namely to phagocytosis by polymorphonuclear leucocytes. From the scarcity of extracellular organisms

one may think that the intracellular fluids are not suitable for an active multiplication of *Brucella*, exception is made of zones where the tissues have been subject to considerable damage. In these zones one may find extracellular *Brucella* in large numbers. One would consider that the organism could be placed together with true intracellular parasites, but this is not the case since *Brucella*

have its own enzyme systems with which it is able to use rather ordinary material for its metabolism.

Considering as likely that in man *Brucella* grow in a manner similar to that which has been observed in the experimental infection, the pathogenesis of the disease seems to us better understood.

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Thromboplastic Action of Plasma Protease (*Tryptase*).^{*}

JOHN H. FERGUSON, BURTON L. TRAVIS, AND EARL B. GERHEIM.

From the Department of Physiology, University of North Carolina, Chapel Hill, N.C.

Indirect evidence, based on experimental analogy with trypsin, papain, etc. was reviewed¹ in support of the theory that blood coagulation normally involves natural plasma protease (*tryptase*¹ or *plasmin*² or *fibrinolytic enzyme*³) apparently identical with the agent responsible for fibrinogenolysis, fibrinolysis, and other proteolytic phenomena. Plasma tryptase resembles pancreatic trypsin in many respects, especially relevant to these problems, but does differ in 1. origin,⁴ 2. kinase-specificity,³ and 3. other important ways.² The following experiments confirm some similarities and lead to *direct* evidence that plasma tryptase can participate as a

"thromboplastic" factor in the blood-clotting system.

Reagents. In the first paper⁵ of this series, on the activation and inhibition of the fibrin(ogen)olytic protease (tryptase) in certain plasma fractions, are described: 1. Borate buffer, pH = 7.7, *viz.* 45 vol. 2.5% H_2BO_3 , 45 vol. 0.5% NaCl, 10 vol. 4% $Na_2B_4O_7 \cdot 12H_2O$, used as solvent and diluent (to constant volume) throughout; 2. Harvard (courtesy Drs. E. J. Cohn, J. T. Edsall, and colleagues) human plasma Fraction-I (H.F.), which contains about 60% fibrinogen, a trace of active protease (tryptase) and considerable enzyme-precursor (tryptogen) that can be activated by a suitable kinase (Table I. A); 3. Armour's (courtesy Dr. J. B. Lesh) enzyme-free⁶ bovine plasma Fraction-I (B.F.), of about the same fibrinogen content as H.F.; 4. Thrombin (THR.), a 1% soln. of lyophilized rabbit "hemostatic globulin," Lederle's (courtesy Dr. I. A. Parfentjev); 5. Streptokinase (STREP.), the streptococcal material (Garner and Tillett, 1934) of which a 1% extract is an excellent kinase-type activator of the plasma protease; 6. Crystalline trypsin-inhibitors, protein (or polypeptide) in nature, from pancreas (T.I.)

^{*} This report is the second of a series of investigations on "Enzymes and enzyme-inhibitors, in relation to blood coagulation and hemorrhagic diseases," aided by a grant from the John and Mary R. Markle Foundation.

The human plasma fractions used in this work were prepared from blood collected by the American Red Cross under a contract between the Office of Scientific Research and Development and Harvard University.

¹ Ferguson, J. H., *Science*, 1943, **97**, 319.

² Christensen, L. R., and Macleod, C. M., *J. Gen. Physiol.*, 1945, **28**, 559.

³ Kaplan, M. H., *J. Clin. Invest.*, 1946, **25**, 331;

337, *et seq.*

⁴ Tagnon, H. J., *Arch. internat. physiol.*, 1942, **51**, 472.

⁵ Ferguson, J. H., Travis, B. L., and Gerheim, E. B., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 285.

TABLE I.

Thromboplastic Action of Plasma Protease (Trypsin) Compared with Pancreatic Trypsin.
A. Preparation of Activators. Timing of Fibrinogenolysis. Clotting-time test at 24°C.

	Mixture (cc)	¼ min.	¼	½	¾	1	1½ hr (incub. at 39°C for lysis)
		sec.	sec.	sec.	sec.		
A.	4.0 H.F. 0.4 STREP.	6	15	45	85	+	∞
B.	4.0 H.F. 0.4 TRYP. (20 unit)	6	21	51	90	+	∞
C.	4.0 H.F. 0.4 buffer	heat-defibrination at 45°C for 3 min.; filtered through glass wool.					

B. Prothrombin Activation Tests. Recalcified PRO.-A.

Clotting-times (c.t.), in seconds, at 24°C. pH = 7.7 (borate buffer) for 0.5 cc B. F. + 0.25 cc T, sampled at stated incubation periods (i.t.).

T.	Activator	¼ min.	¼	1	2	6	18	24 hr	4 day	7 day	(i.t.)
		sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	
1.	None	268	228	195	163	110	72	55	28	13	(c.t.)
2.	Mixture C	307	247	207	172	98	68	55	28	12	
3.	" A	324	132	86	62	45	36	28	20	12	
4.	" B	335	58	40	27	13	8½	7	5	5	
5.	40-unit TRYP.	—	5	5	5	6	15	19	100	—	

and soybean (S.B.I.), 0.2%, courtesy Dr. M. Kunitz (Rockefeller Institute, Princeton). The present studies also use 7. a Harvard (courtesy Dr. J. T. Edsall) human plasma protein fraction (III-3), 0.1%, possessing proteolytic and thrombic properties; 8. Purified prothrombin (PRO.), 0.2%, from lyophile-dried bovine preparations supplied through the courtesy of Dr. W. H. Seegers⁶ (Wayne Univ., Detroit): PRO.-A being stated to yield 15,200 thrombin units per mg tyrosine N. while PRO.-B is somewhat less purified (see below); 9. Pancreatic trypsin (TRYP.), from 2% stock solution (in glycerol-borate) prepared from commercial (Fairchild Bros. and Foster) trypsin, and diluted to 40 or 20 "unit" strength (1 cc = 1 mg = 100 "units," by fibrinogenolytic test);⁷ 10. Rabbit brain thromboplastin (tpln.), 0.5%, from a commercial (Difco) preparation, devoid of significant proteolytic activity; 11. M/10 CaCl₂(Ca).

Prothrombin. Data on the properties, especially activation and stability, of Seegers' purified prothrombin preparations will be reported in detail elsewhere, but it is relevant

⁶ Seegers, W. H., Loomis, E. C., and Vandembelt, J. M., *Arch. Biochem.*, 1945, 6, 85.

⁷ Ferguson, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1943, 52, 243.

to note: 1. some active thrombin is present and increases on standing at room temperature in borate buffer solutions (Table II, 6); 2. there is a very slow (increased) activation to thrombin on adding calcium salt (Table I, B, 1; Table II, 1), which, in comparison with the prompt and complete activation by Ca + tpln. (Table II, 2), shows either an ultimate reaching of the same optimum in 2-7 days (PRO.-B) or incomplete activation even after a week (PRO.-A), although still capable of reaching the optimum on the subsequent addition of thromboplastin. The obvious suggestion is that the prothrombins are contaminated with small but significant traces of thromboplastic factor (? phospholipid): 3. activation by 1/10 vol. of 40-unit trypsin (and Ca) to optimal thrombic activity in about ½ hr., followed by weak thrombinolysis (Table I, B, 5).

Fibrinogenolysis test. When the natural tryptase in H.F. (human plasma fraction-I) is activated by streptokinase and the fibrinogenolysis⁵ followed by the lengthening clotting-times of 0.5 cc samples added to 0.5 cc thrombin (THR.), after successive incubation periods of the lytic mixture, as illustrated in Table I, A, mixture A, the data show close similarity to lysates of H.F. and weak pancreatic trypsin (1/10 vol. of 15-20

TABLE II.
Inhibition of Tryptase-Activation of Prothrombin.

5 cc vol. of thrombic mixtures (T), containing 2 cc PRO-B + 0.25 cc Ca and activators \pm inhibitors noted, incubated at 24°C, pH = 7.7, for periods stated (i.t.). Clotting-times (sec.): 0.25 cc T + 0.5 cc B.F.

T.	Activator	Inhibitor	$\frac{1}{4}$ min.	$\frac{1}{4}$	$\frac{1}{2}$	1	2	6	24 hr	7 day (i.t.)
			sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.
1.	(Ca only)	0	218	167	133	87	45	30	13	$\frac{1}{4}$ (c.t.)
2.	tpln. (0.25 cc)	0	78	4	4	4	4	4	4	$\frac{1}{4}$
3.	III-3 (2.0 ")	0	77	20	14	10	10	9	8	$\frac{1}{4}$
4.	III-3 (2.0 ")	T.I. (0.75 cc)	71	61	39	25	18	11	9	5
5.	III-3 (2.0 ")	S.B.I. (0.75 cc)	75	92	100	108	194	220	150	45
6.	PRO + buffer (No Ca or III-3)		800	610	555	360	210	125	58	12
7.	III-3 + Ca (No PRO.)		78	—	—	—	—	—	87	—

unit TRYP.), as in mixture B. The lysates from these mixtures, as used in the subsequent prothrombin activation studies, obviously represent very small amounts of proteolytic enzyme.

Tryptic activation of prothrombin (Table I, B). Thrombic mixtures (T) consisting of 2 cc PRO-A, 0.25 cc Ca, and cited activators (to 5 cc vol.) are sampled after successive incubation periods (i.t.), the 0.25 cc samples being added to 0.5 cc fibrinogen (B.F.) and the clotting-times (c.t.) noted. The shortening c.t. denotes increasing amounts of thrombin formation. This method has long been used in our laboratories for quantitative and qualitative study of the first phase of blood-clotting.⁸

The $\frac{1}{4}$ min. test, in all series, shows the trace of thrombin originally present in the particular prothrombin solution used. It is of very little significance even compared to the very slow and incomplete activation by Ca-salt alone, as shown in T₁. Series T₃ and T₄, on the other hand, show a definite "thromboplastic" acceleration of thrombin formation by the lysates containing tryptase (A) and trypsin (B), respectively. To rule out the possibility that H.F. might contain some thromboplastic factor other than tryptase, mixture C was prepared by simple heat (56°C) defibrination. Series T₂ corresponds closely to the T₁ control.

Other data. STREP. (*streptokinase*), itself, in repeated tests, is found to be devoid

of proteolytic, thromboplastic, or thrombic effects. Several other protease-containing plasma products, some of dog and bovine, as well as human, origin, also show the "thromboplastic" action noted in Table I, B. One Harvard human plasma fraction (III-3) is a potent protease but also possesses thrombic activity. This complicates the experimental analysis but, with appropriate controls, serves to show essentially the same phenomena, as illustrated in Table II. Included in these data are some typical results of the addition of known *trypsin-inhibitors* (T.I. and S.B.I.)

Inhibition of "thromboplastic" action of plasma protease III-3 (Table II). PRO-B, used in these tests, forms thrombin "spontaneously" (T₆) at a somewhat greater rate than the purer product (PRO-A), and Ca (T₁) gives maximal activation in 7 days, the 4-sec. clotting-time ultimately reached being identical with the stable value attained in 10-15 min. in the additional presence of brain thromboplastin (T₂). The protease III-3 is markedly "thromboplastic" (T₃), as shown by comparison with controls T₁ and T₇. The last shows only a weak initial thrombic action of III-3 alone which, if anything, is lessened after 24 hr. Pancreatic trypsin-inhibitor (T.I.) is clearly (T₄) able to inhibit a considerable amount of the thromboplastic effect of the protease. Soybean trypsin-inhibitor (S.B.I.) is even more effective (T₅), but the later tests suggest that part of this inhibition may be directed not only against the protease (III-3) but also against the natural thromboplastic factors undoubtedly operating in T₁ and T₆.

⁸ Ferguson, J. H., *Ann. N. Y. Acad. Sci.*, 1947, in press.

Fibrinolysis, etc. Lysis of the fibrin clots in A and B (Table I, A) occurred overnight, as did T_2 (Table I, B) and T_3 (Table II). This confirms the fibrinolytic actions of natural plasma protease. Fibrinolysis was absent, even after 7 days at 37°C, in the enzyme-free controls and in the presence of S.B.I., while it was delayed for 3-4 days by the cited amounts of T.I. Thrombinolysis requires further study, but did not occur with the proteases used in the present study, except trypsin (Table I, T_3). This could be a matter of enzyme strength, however.*

The absence of significant prothrombinolysis (*cf.*⁹), due to choice of very weak enzyme

preparations, is apparently the key to the successful demonstration of these important "thromboplastic" effects.

Summary. The natural protease (*tryptase*) in several plasma protein fractions resembles a weak pancreatic trypsin in its fibrinogenolytic, fibrinolytic, and "thromboplastic" effects. These actions are inhibited by crystalline trypsin-inhibitors from pancreas and soybean. The clear demonstration of thromboplastic action in these quantitatively-controlled tests is direct proof of participation of plasma tryptase in the blood-clotting system.

* More recent tests show that a sufficiently potent tryptase is thrombinolytic.

⁹ Seegers, W. H., and Loomis, E. C., *Science*, 1946, 104, 461.

15776

Chronic Oral Toxicity of Alpha-Naphthyl Thiourea.*

O. GARTH FITZHUGH AND ARTHUR A. NELSON.

From the Division of Pharmacology, Food and Drug Administration, Federal Security Agency, Washington, D.C.

Richter¹ proposed the use of alpha-naphthyl thiourea (usually abbreviated ANTU) as a specific poison for the control of Norway rats because of its high toxicity to rats and its relatively low toxicity to all other species tested. Its emetic property protected dogs in most cases. Although Richter showed that animals became tolerant to acute doses of ANTU, the experiments of McClosky and Smith² indicated a cumulative action with successive doses to rabbits and cats. In experiments conducted for short periods of time

the chronic effects of ANTU paralleled closely those obtained with thiourea and 2-thiouracil.¹ Because of Richter's report of tolerance to ANTU the present study was undertaken to determine the effects of small amounts of ANTU ingested during the lifetime of the rat.

Experimental. Groups of 18 male weanling rats (21 days) from our colony of Osborne-Mendel strain were started on each of 7 diets containing respectively 0, 50, 100, 200, 400, 600 and 800 ppm ANTU. Ground commercial rat biscuits with 1% added cod liver oil served as the basic diet. The ANTU was mixed with the basic diet by means of a rotary batch mixer. Litter mates were selected and assigned to the various groups according to a randomized design of experiment (balanced incomplete blocks).³ All

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¹ Richter, C. P., *J. A. M. A.*, 1945, 129, 927.

² McClosky, W. T., and Smith, M. L., *Pub. Health Rep.*, 1945, 60, 1101.

³ Fisher, R. A., and Yates, F., *Statistical Tables for Biological, Agricultural, and Medical Research*, Oliver and Boyd, Edinburgh, 1938.

TABLE I.
Mean Gain in Weight of Male Rats Fed Diets Containing ANTU.

Time in mo.	Dosage of ANTU, ppm	No. of animals	Mean gain in wt, g	Standard error of mean, g
3	0	18	324.5	± 8.3
	50	17	315.1	± 7.3
	100	18	262.0	± 9.0*
	200	18	202.9	± 7.7*
	400	17	123.2	± 8.2*
	600	18	105.1	± 7.9*
	800	16	101.8	± 8.1*
12	0	12	498.1	±16.0
	50	16	505.8	± 7.2
	100	15	457.7	±22.9
	200	15	416.3	±15.2†
	400	16	260.6	±28.8*
	600	15	248.5	±11.4*
	800	12	240.3	±14.3*

* $p < .001$

† $p < .01$

animals were kept in individual cages in a room with controlled temperature and humidity and were given free access to their respective diets and water. Body weights and food consumption were determined at weekly intervals. Surviving animals were sacrificed at the end of the 2-year period.

Results. Effects on Growth and Mortality. Growth curves of the experimental groups, with the exception of that for the group on 50 ppm ANTU, deviated markedly from that of the controls. The data on the gains in weight during the fast-growing period of the first 12 weeks (Table I) show that the animals on concentrations of 100 ppm, or more, grew significantly ($p = <.001$) less than those on the control diet. When the gains in weight of the experimental and control animals during the first year were analyzed (Table I), the difference between the animals on 100 ppm ANTU and the controls was not significant ($p = .17$). This fact led to a further analysis of the growth data for the last 26 weeks of the first year. During this period all groups of rats on 100 ppm, or more, ANTU grew slower than the control group; however, the differences in growth between the groups on 100, 200 and 400 ppm ANTU and the controls were not significant. The growth rate of the rats on these concentrations of ANTU had increased so that the value for the retardation of growth had

changed from highly significant at 3 months to nonsignificant during the plateau period. This apparent decrease in toxicity undoubtedly was due in part to the development of a tolerance;¹ however, as we have shown in a similar experiment with 2,2-bis (*p*-chlorophenyl)-1,1,1-trichloroethane (DDT),⁴ it was due in part to the lesser daily intake of ANTU per kg of body weight as the animals increased in size. The reduction in dosage per kg of body weight was produced by the rapid increase in weight of the rats during the fast-growing period without a corresponding increase in food intake. A dislike for the food containing ANTU was evident in the rats on dosages from 400 to 800 ppm. For the first 3 or 4 weeks on the experimental diet these rats ate less per kg of body weight than control rats; later their food intake gradually became normal. After the first 3 months rats on dosage levels of 600 and 800 ppm were consuming daily more ANTU than the acute lethal dose of nontolerant rats of the same age.

All the deaths, except 4 in the group of rats on 800 ppm ANTU, which occurred during the first year of the experiment were caused either by respiratory infections or by middle ear disease. The 4 deaths in the group on 800 ppm appeared to be a direct

⁴ Fitzhugh, O. G., and Nelson, A. A., *J. Pharm. Exp. Therap.*, 1947, 80, 18.

effect of the ANTU; the animals were small and showed other signs of ANTU poisoning. At 18 months, although fewer animals were living in each group on 200 ppm, or more, ANTU than in the control group, no group had a death rate significantly different from that of the control. At the termination of the experiment the differences appeared to have a definite break at 200 ppm; however, because of the small number of animals the differences at 200 and 800 ppm ANTU were not significant. When the 4 groups from 200 to 800 ppm ANTU were compared jointly with the 3 groups which had an apparent normal death rate, namely, the groups on 50 and 100 ppm ANTU and the control, the difference in mortality rate was found to be highly significant.

Pathology. Autopsy was done on nearly all the 126 rats, and from 83 of them hematoxylin-eosin stained paraffin sections of various tissues were made for microscopic study. Lung, heart, liver, spleen, pancreas, stomach, small intestine, colon, kidney, adrenal, testis and thyroid were sectioned routinely. In addition, sections of the following structures were obtained from the number of animals given: Parathyroid, 39; bones and muscles of knee joint region, 31; skin, 11; lymph nodes, 10; deformed leg and/or foot, 6; eyelids, 6; urinary bladder, 3; brain, 2. Perls' reaction for ferric iron was used on a number of sections to check the nature of certain pigments.

Externally, the rats were stunted and showed spectacle eyes, thinning and coarsening of the hair with increased prominence of the guard hairs, and deformities of the legs and feet (Table II). Internally, there were no characteristic changes, but some non-specific ones were seen, in addition to those resulting from inanition. Slight or moderate relative or absolute enlargement of the spleen was noted 14 times, most frequently (6 instances) at 200 ppm. Dark semifluid material in the stomach or dark spots on the glandular stomach mucosa were noted 28 times, which together with 4 additional instances of focal hemorrhagic necrosis discovered microscopically make up the 32 listed

TABLE II.
Incidence of the More Frequent Pathological Findings.

Dosage ANTU ppm	No. of animals	Gross findings					Microscopic findings				
		Unarction	Spectacle eye	Hair loss	Leg deformity	Focal stomach hemorrhages	No. examined micro- scopically	Hyaline liver	Calcified casts in kidney	Thyroid hyper- plasia	Splenic hyper- plasia
800	18	13	14	9	2	9	15	5	10	9	7
600	18	8	13	8	3	8	13	5	7	7	8
400	18	7	15	13	3	9	12	2	2	4	8
200	18	5	12	2	0	3	12	3	1	2	7
100	18	1	2	0	0	1	10	0	0	1	2
50	18	1	0	0	0	1	15	1	1	1	5
0	18	1	0	0	0	1	6	0	0	0	0

in Table II. In 7 animals, 5 of which were in the 800 and 600 ppm groups, the thyroid showed questionable or slight generalized enlargement.

The hair changes were most noticeable on the nose and on the rear half of the body and were related in degree to the dosage of ANTU. The cessation of hair growth was present in all rats surviving for the first year on 400 to 800 ppm ANTU and in 2 rats on 200 ppm. Spectacle eyes occurred in all rats on dosages from 200 to 800 ppm ANTU. Changes around the eyes were noticed first in animals on 800 ppm ANTU after about 6 months on the experimental diet, and later they occurred in the animals on lower dosage levels. The 3 instances of spectacle eyes in rats on 100 ppm ANTU appeared after 80 weeks. Sections of skin and eyelids showed mainly atrophy of the various structures. The epithelium was reduced in thickness. Hairs and sebaceous glands were reduced both in number and size. Chronic inflammatory cellular exudate was present in some of the eyelid sections, but not in the skin sections.

Hyperplasia of the thyroid was present microscopically (as judged from increased height of the epithelium and decreased amount of colloid) in about half the animals on 800 and 600 ppm ANTU, and in decreasing frequency at lower dosage levels, as shown in Table II. All the 24 instances of thyroid hyperplasia were of slight degree, except for 3 of slight-moderate and 1 of moderate degree. In a few instances there were papillary ingrowths and heapings of the follicular epithelium which might indicate that the present slight degree of hyperplasia had been somewhat greater at some time in the past.

Microscopic hyperplasia of the splenic pulp (Table II) was of moderate degree in 13 instances and slight in the remaining 24, with the moderate degree more frequent at the higher than at the lower dosage levels. It is probable that parallelism of splenic hyperplasia with dosage of ANTU is not more marked because of counteraction of hyperplasia at the higher dosage levels by inanition, which tends to cause hypoplasia (atro-

phy) of the spleen. In 24 instances, all at 200 ppm or above, a relative increase in the percentage of myeloid cells in the splenic pulp was noted; these spleens included atrophic as well as hyperplastic ones.

Hyaline change in the cytoplasm of the centrolobular hepatic cells (Table II) was noted in 16 instances, all graded as slight or minimal in degree except for 2 of moderate and 1 of slight-moderate degree. The lesion consisted of increased oxyphilia and slight to moderate hyalinization of the cytoplasm of the centrolobular hepatic cells, with a minimal increase in cell size. It resembled the lesser degrees of the typical lesion in the rat liver caused by the feeding of DDT,⁵ but did not show the peripheral segregation of large basophilic cytoplasmic granules and the distinct increase in cell size present in many of the DDT rats. Otherwise, we have not previously noted this lesion. Two rats showed focal necrosis of the liver, an incidence to be expected even in untreated animals. There was no vacuolation of the hepatic cells to indicate hydropic or fatty degeneration.

A slight or even moderate decrease in the thickness of the adrenal cortex was seen in most animals at the higher dosage levels, and less frequently with decreasing dosage level through the 200 ppm group; it was not distinct below that level. There did not appear to be a decrease in the number of cortical cells; the cells were simply smaller than usual, having lost most of the foaminess of the cytoplasm normally present in greater or lesser degree. Fat stains were not done. This change has not been noted previously in simple chronic inanition. The adrenal medulla was not altered.

Calcified tubular casts were found in the renal medulla in very small to moderate numbers (numerous in 2 instances at 800 ppm) and their incidence was proportional to dosage, as shown in the table. They first appeared at 35 weeks. They caused little or no renal tubular destruction. The kidneys otherwise showed nothing of note.

⁵ Nelson, A. A., Draize, J. H., Woodard, G., Fitzhugh, O. G., Smith, R. B., Jr., and Calvery, H. O., *Pub. Health Rep.*, 1944, **59**, 1009.

Foci of superficial terminal hemorrhagic necrosis of the glandular mucosa of the stomach are rather frequently seen in rats found dead, especially after a short period of treatment (*i.e.*, with a highly toxic agent). However, the incidence (Table II) in ANTU rats at the higher dosage levels was greater than usual, and the lesions were found in sacrificed animals as well as in those found dead. A sharp drop in incidence below 400 ppm will be noted. The lesion was generally not massive.

Bone changes (upper half of tibia and lower half of femur), apart from the peculiar deformities noted below, consisted in an increase in the number of trabeculae of spongy bone, an increase to a lesser extent in their thickness, and irregularity and slight thinning of the cortical bone. Sometimes a small portion of the spongy bone had the appearance (increased oxyphilia and decreased density) of being newly formed. In some animals small to moderate amounts of fibrous tissue surrounded some of the spongy bone and encroached on the marrow. Few osteoblasts or osteoclasts were seen, suggesting a slow tempo or chronic nature of these changes.

The changes in the 31 bone marrows sectioned were complicated by the associated ones in the bone, but generally speaking slight myeloid hyperplasia was frequent at the higher dosage levels, less frequent at 200 ppm, and absent below that level. On the whole the marrow in the epiphyses was not as hyperplastic as that in the shafts. The remarks about inanition in connection with the spleen also apply here. The ultimate cause of the hyperplasias in the spleen and bone marrow is uncertain.

Deformities of the legs and feet were noted after the 40th week in 8 animals receiving 400 or more ppm ANTU. There were contractures of the extremities, so that the affected animals had the appearance of walking on their heels and elbows, combined with twisting of the feet or legs; sometimes the paws would face backwards. Chronic granulomas of the feet were more severe and the incidence was distinctly higher in treated

animals than usually have occurred in similar experiments with other substances.

Structures unchanged from their appearance in the controls by the feeding of ANTU were lung, heart, lymph nodes, proventriculus, small intestine, colon, and parathyroid. Pancreas and testis differed only in showing nonspecific changes from inanition. Pigmentation in spleen and adrenal was as generally seen in adult rats. The incidence of middle ear infection, chronic pneumonia, and various spontaneous tumors was not increased.

Discussion. Both the hair changes and the spectacle eyes generally are considered to be caused by nutritional deficiencies. Richter¹ has reported that feeding cystine prevents the cessation of hair growth. Circumocular alopecia, or the "spectacle eye condition," has been reported to occur in biotin deficiency⁶ and in inositol deficiency;⁷ however, this condition often is associated with dermatitis elsewhere and is not necessarily a sign of a single deficiency of a member of the vitamin B complex.⁸ Many of the rats on the higher dosages of ANTU showed inanition to a marked degree, and therefore complex nutritional deficiencies were probably present.

Destruction of bone and joint tissues in the feet and lower legs by suppurative inflammation and the reactive processes consequent thereto appeared to be the underlying cause of the deformities of the legs and feet. The subsequent contractures and twisting are accounted for in the same way that they occur in human cases unless they are prevented. The chronic granulomas of the feet, which are occasionally seen in our rats, presumably resulted from the trauma of the cage wires. It seems reasonable to assume that the various nutritional, endocrine and osseous upsets produced by feeding ANTU caused the tissues of the feet to be a more favorable location than they are usually for

⁶ Nielsen, E., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1941, **48**, 349.

⁷ Paveek, P. L., and Baum, H. M., *Science*, 1940, **92**, 502.

⁸ Sullivan, M., and Nicholls, J., *Arch. Derm. and Syph.*, 1942, **45**, 917.

bacterial growth. Except where involved by adjacent inflammatory processes, the muscles showed surprisingly little change. The lack of muscle changes rules out to some extent a vitamin E deficiency.

Summary. When ANTU was fed chronically to rats for 2 years in concentrations of 50 to 800 ppm in the diet, the concentrations from 100 to 800 ppm were toxic. Externally, the rats were stunted and showed spectacle eyes, thinning and coarsening of the hair and deformities of the legs and feet. Histo-

pathological changes consisted of hyperplasia of the thyroid, hyperplasia of the splenic pulp, hyaline changes in the cytoplasm of the centrolobular hepatic cells, a slight to moderate decrease in the thickness of the adrenal cortex, calcified tubular casts in the renal medulla, slight myeloid hyperplasia of the bone marrow, and slight modifications in bone structure. The degree of injury in each case was related to the concentration of the ANTU. The concentrations of 600 and 800 ppm ANTU produced a marked tolerance.

15777

Separation of Two Mutually Antagonistic Chromatophorotropins from the Tritocerebral Commissure of *Crago*.

FRANK A. BROWN, JR., AND IRVING M. KLOTZ.

From the Departments of Zoology and Chemistry, Northwestern University, Evanston, Ill., and the Marine Biological Laboratory, Woods Hole, Mass.

From the time of Koller's original contention that he had discovered in the rostral region of the thorax of the shrimp, *Crago*, a new endocrine source, producing a principle which blackened white-adapted specimens,¹ repeated attempts to confirm this conclusion have been generally unsuccessful at the hands of other investigators. More recently it has been shown that the circumoesophageal connective region,² or more specifically the tritocerebral commissure, of *Crago*³ possesses very potent chromatophorotropic activity. Extracts of the commissures in sea-water, injected into eyestalkless specimens produce within 5 minutes striking lightening of the body and simultaneously blackening of the tail-fin (telson and uropods). It has been postulated upon the basis of such observations as the following, that the tritocerebral commissure of *Crago* possesses 2 mutually antagonistic principles for general body-coloration: (1) extracts of commissures from

different individuals show variation in their relative influences in body-lightening and tail-fin-darkening, at times even darkening the body instead of lightening it,³ (2) stimulation of the stubs of the eyestalks in eyestalkless animals results in body-lightening and tail-fin-darkening when the stimulus is strong, and, overall blackening if the stimulus is weak,⁴ (3) comparative studies of crustacean nervous systems show some to possess only body-lightening activity (e.g. the crab, *Uca*), others predominantly body- and tail-fin-darkening activity (e.g. the lobster, *Homarus*).⁵

A clear proof, however, that the observed results of the action of extracts of *Crago* tritocerebral commissures are due to 2 hormones (1) a body-blackening hormone, and (2) an antagonistic body-lightening one obviously requires the chemical fractionation of the *Crago* commissure into 2 portions, each possessing only one of these 2 actions.

¹ Koller, G., *Z. vergl. Physiol.*, 1928, **8**, 601.

² Brown, F. A., Jr., and Ederstrom, H. E., *J. Exp. Zool.*, 1940, **85**, 53.

³ Brown, F. A., Jr., *Physiol. Zool.*, 1946, **19**, 215.

⁴ Brown, F. A., Jr., and Wulff, V. J., *J. Cell. Comp. Physiol.*, 1941, **18**, 339.

⁵ Brown, F. A., Jr., and Saigh, L. M., *Biol. Bull.*, 1946, **91**, 170.

TABLE I.
Fractionation of Principles of *Crago* Commissure by Drop Method.
Absolute Ethyl Alcohol as Extracting Solvent.

Extraction No.	No. of drops of solvent	Quantity of sea water to take up residue cc	Size of injection, cc	Assay	
				Body	Tail
1	5	.06	.03	—4	0
2	5	.06	.03	—4	0
3	5	.06	.03	—3	0
4	5	.06	.03	—1	0
5	5	.06	.03	—1	0
6	—	.06*	.03	—2 i†	+4
				+4 f	

* Water was added directly to the slide and the smeared tissue was scraped off and thoroughly mixed with the liquid.

† i means initial; f means final.

TABLE II.
Fractionation of Principles of *Crago* Commissure by Equilibrium Method.
Absolute Methyl Alcohol as Extracting Solvent.

Extraction No.	Quantity of solvent in test tube, cc	Quantity of sea water to take up residue, cc	Size of injection, cc	Assay	
				Body	Tail
1	.50	.06	.03	—3	0
2	.50	.06	.03	0	0
3	—	.06	.03	—1 i†	+4
1	.50	.06	.03	—3	0
2	.50	.06	.03	—1	0
3	.50	.06	.03	0	0
4	.50	.06	.03	0	0
5	—	.06*	.03	0 i†	+3
				+1 f	

* Water was added directly to the slide and the smeared tissue was scraped off thoroughly and mixed with the liquid.

† i stands for initial; f stands for final.

Investigations of the solubility of the active commissural material in organic solvents³ indicated that one of the factors is preferentially soluble in polar organic substances, such as ethyl alcohol. A fractionation method based on an extraction procedure was thus suggested. Craig has shown⁶ that the method of successive extractions can be used very successfully in fractionating substances of biological interest. Unfortunately his procedure deals with quantities of the order of 20 μ g or more. A rough measurement of the size of the glandular tissue of the commissure as determined from histological serial sections indicated that the quantity of active principle available must be much smaller than even one microgram. Consid-

ering the region of the commissure possessing the gland as approximately a cylinder, one finds for an average specimen a diameter of about 0.005 cm, a length of about .05 cm, and hence a volume of about 1×10^{-6} cc. Assuming a density of about 1, one obtains 1×10^{-6} g for the mass of this structure. A very generous estimate of the actual glandular tissue in the commissure would be about 25%. The concentration of active substance within the glandular tissue could hardly exceed 33% so that a mass of 0.1 μ g is probably an upper limit to the total quantity of active material present. It is this 0.1 μ g which must be fractionated into 2 components.

Experimental. A commissure was carefully excised from a medium sized (1 g) specimen of the shrimp, *Crago*, as previously described³

⁶ Craig, L. C., *J. Biol. Chem.*, 1944, 155, 519.

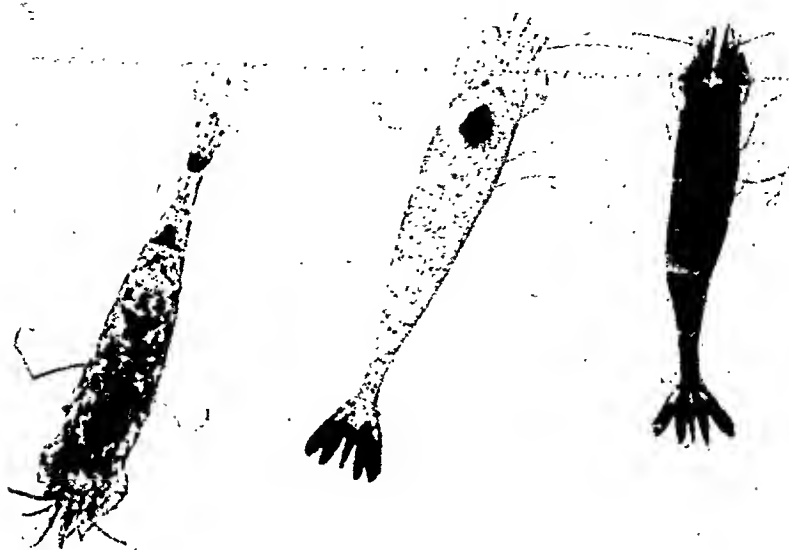


FIG. 1.

From left to right: 1. A sea-water-injected eyestalkless *Crago* (control); 2. an eyestalkless specimen receiving an injection the equivalent of one-sixth of the sea-water-soluble contents of one tritocerebral commissure; 3. an eyestalkless specimen receiving an injection the equivalent of one-sixth of the alcohol-insoluble contents of a tritocerebral commissure. The injections were made about nine minutes before the photograph was taken. All 3 animals were matched and resembled specimen one at the beginning of the experiment.

and deposited on a microscope slide or cover-glass. The surrounding water was allowed to dry for about 2 minutes. The specimen was then smeared over an area of about 0.5 cm^2 with the aid of a blunt glass rod or cover-glass. Examination under the microscope indicated that the maximum thickness of the smeared specimen was about 5μ and that numerous valleys and ridges were present to facilitate penetration by the solvent.

Two alternative procedures were used to carry out the extraction. One consisted of dropping the organic solvent onto the tilted slide bearing the smeared specimen and collecting the liquid on a depression slide. The liquid was collected in groups of 5 or 10 drops. An interval of a few minutes was allowed between each group so that the specimen slide could dry.

This procedure was found to have the objectionable feature, particularly with very volatile solvents, that rapid evaporation of the solvent cooled the slide appreciably and led to the condensation of water vapor on the specimen. The water could dissolve both

active principles and hence nullify the effort at fractionation. This difficulty was overcome at least partially by drying between groups of drops, but some doubt always remained.

An additional objection to this drop procedure lies in the difficulty of carrying out a physico-chemical analysis of the results since the establishment of equilibrium between smear and solvent can be seriously questioned.

To overcome the objections mentioned a modified approach was also used. The cover-glass containing the smear was cut down to approximately the area occupied by the tissue and then inserted into a 3 cc test tube. 0.50 cc of solvent was added to the tube, which was then stoppered and shaken carefully for about 10 minutes. The closed tube minimized evaporation and the long time of contact between tissue and solvent insured the attainment of equilibrium.

With either procedure the solvent was evaporated in the open air and the residue was taken up in sea water. The sea-water

solution was used in all injections.

The method of assay has been described previously.² A scale from 0 to 4 has been established to estimate the intensity of a given effect. A negative sign indicates a lightening with respect to an appropriate control, a positive sign indicates a darkening.

Results. A typical experiment using the drop technic is summarized in Table I and an example of the test-tube method is illustrated in Table II. In both cases it is quite clear that the body-lightening factor (CBLH) can be separated without any significant content of the tail-darkening principle (CDH). The solvent used in the experiment of Table I, however, is not exceedingly volatile. Using isopropyl alcohol as the extracting solvent, a cleaner separation was effected. With methyl alcohol, which vaporized very rapidly, assays usually show a slight amount of tail-darkening in the col-

lected drops of solvent. It was quite obvious that water was condensing on the slide during the dropping of the methyl alcohol and the water must have carried along a little of the tail-darkening factor, for when the closed-tube technic was adopted (Table II) none of the darkening factor appeared in the alcohol extracts. Apparently the darkening factor has no significant solubility in the organic solvents used.

Fig. 1 illustrates the action of a sea-water extract of a tritocerebral commissure which contains both CBLH and CDH, and the action of only the alcohol-insoluble fraction of the commissure which contains CDH alone.

Summary. The tritocerebral commissure of the shrimp, *Crago*, has been chemically fractionated to separate 2 mutually antagonistic chromatophorotropins influencing body-coloration, a general darkening one (CDH) and a body-lightening one (CBLH).

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Radioautographs in Which the Tissue is Mounted Directly on the Photographic Plate.

TITUS C. EVANS.*

From the Radiological Research Laboratory, Columbia University, New York City.

In early methods of determining the location of radioactive elements in tissue, the microscope slide bearing the tissue section was placed face down on a photographic plate.¹⁻⁴ After a suitable exposure to the radiation the plate was removed and developed. The resulting image, or autograph, was then compared with the tissue section (after staining etc.) to locate the radioactive

material. The efficacy of this method is limited by (1) lack of sharpness and (2) difficulty in matching the autograph with the corresponding histologic detail in the tissue.

Recently, Bélanger and Leblond⁵ have described a method which reduces these objections. The photographic emulsion from a lantern slide is removed and spread over the tissue section. After the radiation exposure the autograph is developed, fixed and washed. The tissue sections are then stained, cleared and the entire preparation mounted in balsam.

For some time the writer had been considering the feasibility of mounting tissue sections directly on the photographic emul-

* It is a pleasure to acknowledge the assistance and encouragement given to the writer by his associates in the laboratory.

¹ Lacassagne, A., and Lattes, C., *J. Radiol. et Elect.*, 1925, 9, 1.

² Hamilton, J. G., Soley, M. H., and Eichorn, K. B., *Univ. California Publ., Pharmacol.*, 1940, 1, 339.

³ Leblond, C. P., *J. Anat.*, 1943, 77, 149.

⁴ Hamilton, J. G., *Radiology*, 1942, 39, 541.

⁵ Bélanger, L. F., and Leblond, C. P., *Endocrinology*, 1946, 39, 8.



FIG. 1.

From left to right: 1. A sea-water-injected eyestalkless *Crago* (control); 2. an eyestalkless specimen receiving an injection the equivalent of one-sixth of the sea-water-soluble contents of one tritocerebral commissure; 3. an eyestalkless specimen receiving an injection the equivalent of one-sixth of the alcohol-insoluble contents of a tritocerebral commissure. The injections were made about nine minutes before the photograph was taken. All 3 animals were matched and resembled specimen one at the beginning of the experiment.

and deposited on a microscope slide or cover-glass. The surrounding water was allowed to dry for about 2 minutes. The specimen was then smeared over an area of about 0.5 cm^2 with the aid of a blunt glass rod or cover-glass. Examination under the microscope indicated that the maximum thickness of the smeared specimen was about 5μ and that numerous valleys and ridges were present to facilitate penetration by the solvent.

Two alternative procedures were used to carry out the extraction. One consisted of dropping the organic solvent onto the tilted slide bearing the smeared specimen and collecting the liquid on a depression slide. The liquid was collected in groups of 5 or 10 drops. An interval of a few minutes was allowed between each group so that the specimen slide could dry.

This procedure was found to have the objectionable feature, particularly with very volatile solvents, that rapid evaporation of the solvent cooled the slide appreciably and led to the condensation of water vapor on the specimen. The water could dissolve both

active principles and hence nullify the effort at fractionation. This difficulty was overcome at least partially by drying between groups of drops, but some doubt always remained.

An additional objection to this drop procedure lies in the difficulty of carrying out a physico-chemical analysis of the results since the establishment of equilibrium between smear and solvent can be seriously questioned.

To overcome the objections mentioned a modified approach was also used. The cover-glass containing the smear was cut down to approximately the area occupied by the tissue and then inserted into a 3 cc test tube. 0.50 cc of solvent was added to the tube, which was then stoppered and shaken carefully for about 10 minutes. The closed tube minimized evaporation and the long time of contact between tissue and solvent insured the attainment of equilibrium.

With either procedure the solvent was evaporated in the open air and the residue was taken up in sea water. The sea-water

tioning are done according to the usual histologic technics. The paraffin ribbons are then cut into strips of 2-4 sections and allowed to expand on the surface of water at 42°C in a small petri dish. This is dropped into a larger bowl which allows the sections to float out into cold water. The photographic plate (lantern slide, or film not sensitive to red light) is then slipped under the tissue sections in dark room, in front of red safelight, one corner of the section is held against the emulsion of the plate and the plate plus the tissue removed from the water. The preparation is then dried in front of a fan and placed in a light-tight box for exposure to the radiation. Before developing the photographic plate the paraffin is removed with xylol and when this has evaporated the plate is developed in Eastman D-11 or D-72 solution. After fixation in acid hypo, the preparation is washed and allowed to dry. If it is desired that the background (and also the image) be reduced, this can be done by dipping the preparation, before the tissue is stained into a 0.2% permanganate solution.⁶

The tissue is stained with Harris' hematoxylin (over stain, acid water, alkaline water) and aqueous eosin, then dehydrated, cleared and mounted in permount or balsam. The plate should be dried as often as possible. Swelling and reticulation of the emulsion must be avoided.

Several methods of avoiding heavy staining of the photographic film, which is more of a problem than when plates are used, have been employed successfully. One meth-

od is staining *in toto* (acetocarmine, Harris' hematoxylin, or eosin when absolute alcohol is the fixative). Another, is to place ferric alum in with the fixative⁷ in order that the tissue may be selectively stained when the preparation is immersed in iron hematoxylin. Care must be exercised in the second use of ferric alum since it will reduce the autograph as well as the nuclear stain.

Tissue prepared in the usual way may be used to check detail and staining properties. Some of the tissue should also be used for autographs using the older method, to insure that the general distribution of the radioactive material appears to be the same by both methods.

Tissue-autograph preparations offer many advantages over the older method of preparing the two separately. The entire section may be studied simultaneously for histologic detail and for localization of the radioactive element. The resolution is better and studies can be made under either the low or high power of the compound microscope (Plate 1). These findings have aided materially the study of experimental animals, of thyroid adenomas, and of thyroid carcinoma metastases.

Summary. The tissue section containing radioactive material is mounted directly on the photographic emulsion in the dark room. After suitable exposure, the plate is developed, and the tissue stained etc. The preparation may be studied microscopically as the autographic image is in place just below the tissue.

⁷ Kupperman, H. S., and Novack, C. R., *Science*, 1943, 98, 591.

⁶ Liebermann, L. N., Barschall, H. H., *Rev. Sci. Instr.*, 1943, 14, 89.

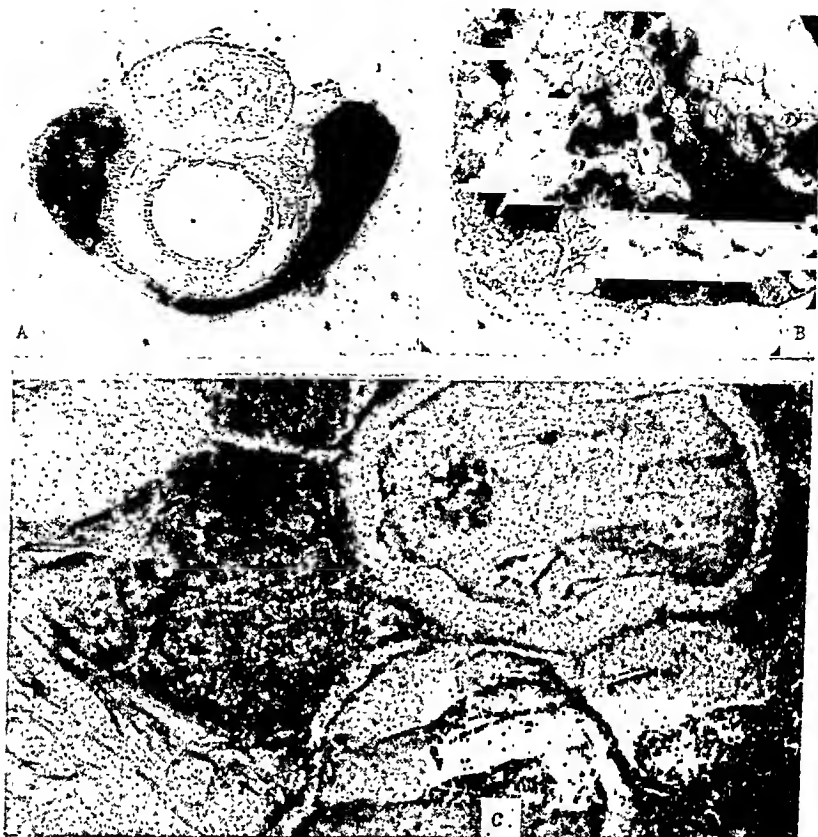


PLATE 1.

Photomicrographs of rat thyroid tissue (hematoxylin and eosin) mounted on the photographic emulsion in which the autograph was later produced directly under the regions containing radioiodine.

A. Thyroid region of newborn rat. Autograph and tissue on commercial film. Magnification $\times 18$. Autograph developed 4 days after tissue containing the radioiodine had been attached. Three days before this babe was born, the mother had been injected with $30 \mu\text{c}$ of I^{131} . The foetus was able to concentrate the iodine in its thyroid, in competition with the mother, as can be seen by the heavy blackening which is limited to the thyroid regions.

B. Portion of thyroid of young adult rat (wt. 171 g) which had been sacrificed 24 hours after an injection of $25 \mu\text{c}$ of I^{131} . Magnification $\times 25$. Medium contrast lantern slide, 6-day exposure. The indication of presence of radioiodine is most pronounced in the smaller follicles.

C. Small region of same thyroid shown in B, $\times 188$. Deposition of the reduced silver is heavy underneath the one triangular follicle, whereas that under the others is only slightly greater than background (*i.e.*, lower left corner).

sion. When the paper of Bélanger and Leblond appeared, their method was tried. The preparations, although better than those obtained with the older methods, were not completely satisfactory. The emulsion was uneven and contained bubbles, the autograph was not sharp enough for high power study, the "fog" was heavy, and staining was complicated by the gelatin over the tissue, which

became colored and lifted away from the slide. No doubt, some of the difficulties were due to inexperience and would be eliminated in time. It seemed advisable, however, to try our first plan, *i.e.* to mount the tissue directly on the plate. This method has been satisfactory and gives much better resolution than was possible formerly.

Fixation, dehydration, embedding and sec-

TABLE I.

Folic Acid Storage in Chick Liver and Muscle with Increasing Dietary Intakes of Folic Acid.

Group No.	Supplement added per 100 g ration 494-K	Avg wt at 4 wk, g	γ of folic acid per g of	
			Liver	Muscle
1	0	130 (6)*	1.07 (.79-1.52)†	.05 (.042-.069)
2	25 γ folic acid	175 (5)	2.35 (1.46-3.08)	.06 (.04-.07)
3	100 γ " "	190 (6)	3.52 (1.97-4.51)	.06 (.05-.08)
4	1000 γ " "	175 (6)	14.7 (11.9-16.4)	.04 (.03-.05)

* Numbers in parentheses refer to number of chicks surviving at 4 weeks.

† Numbers in parentheses give the range of values found.

chicks receiving 200 γ of folic acid per 100 g of our basal ration. The results of these series are summarized in Tables II and III.

Samples taken for analysis were minced in a Potter-Elvehjem homogenizer⁴ or a Waring Blendor. Liver and muscle samples from chicks in series I and II were digested with taka-diastase in pH 4.5 acetate buffer for 24 hours at 37°C since this procedure has been found satisfactory for liberating bound folic acid in these tissues.^{5,6} The other body tissues were likewise treated with taka-diastase and in addition duplicate series were treated in pH 4.5 acetate buffer with a kidney conjugase preparation prepared according to the procedure of Bird *et al.*⁷ No significant differences in liberation of folic acid from the various tissues was noted with either procedure and the values given (Table III) represent an average of values from tissues receiving the 2 liberation procedures. Folic acid was determined with *Streptococcus faecalis* using the medium of Luckey *et al.*⁸ modified by the addition of 1.0 cc of Speak-

man's⁹ salts B per 100 cc of medium.

Results and Discussion. The results of the first series demonstrate that increasing the dietary level of folic acid has little effect on the content of this vitamin in chick muscle tissue since muscle folic acid values remained relatively constant regardless of dietary intake. The folic acid content of the liver, however, increased directly with increased dietary intakes of the vitamin. Liver values were 1.07, 2.35, 3.52 and 14.7 γ of folic acid per g of fresh liver for the 0, 25, 100 and 1000 γ dietary level groups respectively. The values presented here are not intended to represent absolute amounts of folic acid that will be found in chick liver and muscle tissue on similar folic acid intakes on different diets; but they merely represent the relative efficiency of storage of folic acid under the particular standardized conditions used in this series (Series I). The observed increase of folic acid in chick liver as compared to the constancy of chick muscle tissue follows the expected storage of a B vitamin and is in contrast to the increased storage of pantothenic acid in muscle tissue reported by Pearson *et al.*³

Results from the second series indicate that high initial dosages of the vitamin are stored inefficiently in the liver since only 3.6 γ of folic acid per g of liver were present in chicks from Groups 3 and 4 (Table II) 2 days after administration of the final mg of folic acid. Muscle folic acid values remained fairly constant in Groups 1, 2 and 4 but a temporary increase occurred in the injected group. The control group of chicks

⁴ Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1936, 114, 495.

⁵ Cheldelin, V. H., Eppright, M. A., Snell, E. E., and Guirard, B. M., *Univ. Texas Publ.*, 1942, No. 4237, 32.

⁶ Luckey, T. D., Briggs, G. M., Jr., Moore, P. R., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1945, 161, 395.

⁷ Bird, O. D., Robbins, M., Vanderbelt, J. M., and Pfaffner, J. J., *J. Biol. Chem.*, 1946, 163, 649.

⁸ Luckey, T. D., Briggs, G. M., Jr., and Elvehjem, C. A., *J. Biol. Chem.*, 1944, 152, 157.

⁹ Speakman, H. B., *J. Biol. Chem.*, 1923-24, 58, 395.

Storage, Retention and Distribution of Folic Acid in the Chick.*

P. R. MOORE, A. LEPP, T. D. LUCKEY, C. A. ELVEHJEM, AND E. B. HART.

From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison.

The water-soluble vitamins are generally stored and retained by animal tissues to a much smaller extent than the fat-soluble vitamins. High oral intakes of vitamin A are known to produce high concentrations of this vitamin in the livers of rats and once established these stores are available to the animal for long periods of time following the exclusion of vitamin A from the diet.¹ Data on the storage and retention of the B vitamins are limited. Schultz *et al.*² were unable to find a further increase in the body concentration of thiamine after a dietary level of 65 γ of thiamine per rat per day was reached. Pearson *et al.*³ found that an increased dietary level of pantothenic acid had no significant effect on the level of this vitamin in the livers of chicks but did produce increases in the leg and breast muscle.

In the course of our studies on folic acid in chick nutrition we were interested in the storage, retention, and distribution of this vitamin in the chick. Results of these investigations are reported here.

Experimental. Day-old White Leghorn cockerels obtained from a commercial hatchery were maintained in electrically-heated cages with raised screen bottoms. All chicks were fed our folic acid-deficient basal ration 494-K which consists of dextrin 61 g, alcohol-extracted casein 18 g, gelatin 10 g, soybean oil 5 g, salts V 6 g, cystine 0.3 g, thiamine 0.3 mg, riboflavin 0.6 mg, nicotinic acid

5.0 mg, pyridoxine hydrochloride 0.4 mg, calcium pantothenate 2.0 mg, inositol 100 mg, choline chloride 150 mg, biotin 0.02 mg, 2-methyl-1,4-naphthoquinone 0.05 mg, and α -tocopherol 0.3 mg. Each chick received in addition one drop per week of fortified haliver oil containing 120 A.O.A.C. units of vitamin D₃ and 1200 U.S.P. units of vitamin A per drop. Synthetic folic acid (pteroyl glutamic acid) was used in all experiments. Food and water were given *ad libitum*. The chicks were maintained on the above ration for 3 to 4 days, weighed, and chicks within a 10 g weight range were distributed uniformly into the experimental groups.

In the first series groups of 8 chicks each were placed on folic acid levels of 0, 25, 100, and 1000 γ per 100 g of ration. The number of chicks per group was reduced to 6 after the first week by discarding the heaviest and lightest animal from each group. The animals were sacrificed when 4 weeks old and representative samples of liver and muscle tissue were taken from 4 chicks in each group for folic acid analyses. Average weights of the animals at 4 weeks, number of survivors, and folic acid values for the liver and muscle tissues are summarized in Table I.

In a second series, 4 groups of 16 chicks each were placed on the following diets: unsupplemented basal, basal plus 200 γ of folic acid per 100 g of ration, basal plus 1000 γ of folic acid per chick per day for 5 days (total of 5 mg per chick) by intraperitoneal injection, and basal plus 1000 γ of folic acid per chick per day for 5 days orally. Four chicks from each group were taken for folic acid analysis of the liver and muscle tissues at the end of 1, 3, and 5 weeks from the time the supplements were started. In addition, folic acid analyses were made on representative tissues from 3 series of 4- to 5-week-old

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1 Baumann, C. A., Foster, E. G., and Moore, P. R., *J. Biol. Chem.*, 1942, **142**, 597.

2 Schultz, A. S., Light, R. F., Caacas, L. J., and Atkin, L., *J. Nutrition*, 1939, **17**, 143.

3 Pearson, P. B., Melass, V. H., and Sherwood, R. M., *J. Nutrition*, 1946, **32**, 187.

dosages of folic acid were used in the synthesis of a biologically active but microbiologically inactive compound. This view has been taken previously by Wright *et al.*¹⁰ who observed a decrease of folic acid (as measured microbiologically) in incubated rat liver tissue. In any event, low tissue levels of folic acid are utilized efficiently on low folic acid diets. The possibility of storage in some other tissue of the animal seems improbable since analysis of the various body tissues of chicks receiving 200 γ of folic acid per 100 g of diet showed the liver to be the main site of storage.

Results from the second series also demonstrate that high initial doses of orally administered folic acid are used as efficiently as equivalent doses of injected folic acid. Group 4, which received the folic acid supplement by the oral route, gave a growth response at 3 weeks equivalent to that of the injected group (Group 3); and at 5 weeks the growth of the 2 groups plateaued at the same weight (difference of 10 g is not considered significant). These results do not substantiate the findings of Frost and Dann¹¹ who found differences in favor of the injected folic acid but a direct comparison of the data

¹⁰ Wright, L. D., Skeggs, H. R., and Weleh, A. D., *Arch. Biochem.*, 1945, 6, 15.

¹¹ Frost, D. V., and Dann, F. P., *Science*, 1946, 104, 492.

is impossible since different technics and animals were involved.

Liver, kidney, and pancreas were found to have the highest and skin and muscle the lowest content of folic acid; testes, spleen, heart, brain, and lung had intermediate values. The relative content of folic acid in chick liver, heart, and brain agree with the values found by Williams *et al.*¹²

Summary. High oral intakes of folic acid are stored inefficiently in chick livers and have little if any effect on the content of this vitamin in chick muscle tissue. Chicks given large oral doses of folic acid and then placed on a folic acid-deficient diet grew as well as chicks receiving an equivalent amount of folic acid by injection. The distribution of folic acid in some representative chick tissues has been studied.

We are indebted to Merck and Company, Rahway, N.J., for crystalline vitamins; to Dr. B. L. Hutchings of Lederle Laboratories, Inc., Pearl River, N.Y., for crystalline folic acid; to Abbott Laboratories, North Chicago, Ill., for haliver oil; to Wilson and Company, Inc., Chicago, Ill., for gelatin; to Allied Mills, Inc., Peoria, Ill., for soybean oil; and to E. I. du Pont de Nemours and Company, Inc., New Brunswick, N.J., for crystalline vitamin D₃.

¹² Williams, R. J., Taylor, A., and Cheldefin, V. H., *Univ. of Texas Publ.*, 1941, No. 4137, 61.

15780

Intrasplenic Transplantation of Testes in Castrated Mice*

MIN HSIN LI,[†] CARROLL A. PFEIFFER, AND W. U. GARDNER.

From the Department of Anatomy, Yale University School of Medicine.

Granulosa-cell tumors and luteomas have arisen in intrasplenic transplants of ovaries in castrated male and female mice.^{1,2} The pathogenetic factors responsible for these

ovarian tumors have been assumed to be (1) the capability of the liver to inactivate ovarian hormones, and (2) the increase in the amount of gonadotrophic hormones that

* Aided by grants from the Anna Fuller Fund and the Jane Coffin Childs Memorial Fund for Medical Research.

[†] Anna Fuller Fund Fellow in Anatomy.

¹ Li, M. H., and Gardner, W. U., *Science*, 1947, 105, 13.

² Li, M. H., and Gardner, W. U., *Cancer Research*, in press.

TABLE II.
Folic Acid Content of Liver and Muscle Tissue from Chicks Receiving Various Folic Acid Supplements.

Method and amount of supplementation	Avg weight and folic acid values in γ/g									
	1st week*						3rd week		5th week	
	Avg wt, g	Folic acid in Liver	Muscle	Avg wt, g	Folic acid in Liver	Muscle	Avg wt, g	Folic acid in Liver	Muscle	
0	85	.80 (0.4-1.2)†	.05 (.04-.06)	95	1.1 (0.7-1.1)	.04 (.04-.05)	85	1.0 (0.4-2.1)	.09 (.05-.13)	
200 γ folic acid per 100 g basal diet	90	2.1 (1.2-2.7)	.05 (.04-.06)	215	1.6 (1.2-1.9)	.06 (.05-.08)	410	3.9 (3.6-4.3)	.07 (.07)	
1 mg folic acid per day for 5 days by injection	90	3.6 (2.8-5.0)	.27 (.21-.34)	220	1.2 (1.2-1.7)	.09 (.07-.12)	395	0.6 (.45-.70)	.10 (.09-.16)	
1 mg folic acid per day for 5 days orally	90	3.6 (1.4-5.0)	.08 (.06-.09)	210	0.7 (.5-0.7)	.06 (.05-.07)	385	0.34 (.30-.40)	.12 (.09-.18)	

* Weekly periods are given, based on the following schedule: 1st week, 10-17 days; 3rd week, 27-34 days; 5th week, 44-51 days.

* Weekly periods are given from the time supplementation was started.

† Numbers in parentheses refer to range of folic acid values.

‡ Number of deaths during the 5-week period was 1, 0, 0, and 0 for groups 1, 2, 3, and 4 respectively.

TABLE III.
Folic Acid Content of Tissues from 4.5-Week-Old Chicks.

Tissue	Folic acid in γ/g *	
Liver	3.05	(1.8-4.3)†
Kidney	1.6	(1.0-2.2)
Pancreas	.95	(.4-1.5)
Testes	.6	
Spleen	.28	(.19-.27)
Heart	.21	(.19-.23)
Brain	.19	(.17-.2)
Lung	.17	(.10-.24)
Muscle	.08	(.04-.12)
Skin	.05	(.05-.06)

* Each value represents the average of 6 samples, each of which consisted of the pooled tissue of 2 chicks (total of 12 chicks).

† Numbers in parentheses refer to range of folic acid values encountered.

which received the 200 γ level of folic acid had liver and muscle folic acid values in fair agreement with those obtained in the first series. The failure of the chicks on the 200 γ dietary level of folic acid (Table II) to show the expected increases in liver folic acid values over the groups receiving the 100 γ dietary level (Table I) suggests more efficient storage of folic acid at an early age in the slower growing animals of the first series. No significant decreases in liver folic acid occurred in the basal group and the 0.6 and .36 γ/g drop from the 3rd to the 5th week in Groups 3 and 4 respectively is small compared to the 2.4 and 2.9 γ/g drop that occurred from the 1st to the 3rd week. Further, the increased growth from the 3rd to the 5th week as compared to the 1st to the 3rd week would act to distribute the vitamin over a larger weight of tissue and the decreases in the total amount of liver folic acid would actually be smaller than is indicated by the values given. The folic acid in the basal ration (3 to 4 γ per 100 g) plus that produced from a limited amount of intestinal synthesis would aid slightly in maintaining liver stores. Nevertheless, the ability of the injected and orally administered groups to continue growing in spite of the fact that their liver and muscle tissues were as low in folic acid as the corresponding tissues of the basal group is interesting. Explanations for this discrepancy are lacking, but one possibility is that the initial high

275 days. Testicular grafts were not found, however, in 2 hosts sacrificed at 273 and 296 days after the operation. Microscopically, the testicular grafts were well incorporated with the splenic tissues and blood sinuses although major portions of the grafts were covered only by the splenic capsule. A few layers of proliferating spermatogonia and spermatocytes were observed in the seminiferous tubules of transplants recovered after 63 to 140 days. Sloughing of the spermatogenic cells into the lumina of the tubules, resulting in a disorganized appearance of the seminiferous epithelium, was frequently encountered. A slight increase in the number of interstitial cells of Leydig, as compared to a normal testis, was noted in 3 testicular grafts. Minute vacuoles were present in the cytoplasm of many of the interstitial cells. In testes grafted into the spleens for a period of over 160 days the seminiferous epithelium was generally thin, although the lumina of the seminiferous tubules remained large (Fig. 3). A few large primary spermatocytes containing 2 or more nuclei were noted in many of the tubules. The nuclei in these large primary spermatocytes were usually at the same stage of meiosis or were in a resting stage with a distinct nuclear membrane. Multinucleated masses containing pycnotic nuclei were also noted in some tubules. The interstitial cells were present in the coagulated tissue fluid that occupied the intertubular spaces (Fig. 4). In 2 grafts a few seminiferous tubules were necrotic. Fibrous and splenic tissues invaded parts of these 2 grafts. Of particular interest was the finding of spermatids in a testis 243 days subsequent to intrasplenic transplantation. A small group of spermatids, with at least 3 cells showing late spermiogenesis, and many scattered spermatids with indication of sperm head formation were found in the seminiferous tubules. A large number of secondary spermatocytes was present in this testis (Fig. 5). In all other testicular transplants, however, spermatogenesis usually stopped at the primary spermatocyte stage.

The prostates and seminal vesicles of the castrated male mice with intrasplenic testicular transplants without adhesions were

atrophic. In one mouse with vascularized adhesion of the graft to the left kidney the accessory genital organs appeared normal when examined 99 days after transplantation, and the adrenal glands showed degeneration of the x-zone. Effects of castration were observed in the Bowman's capsules of the kidneys,^{9,10} and in the submaxillary glands.^{11,12}

Among 18 castrated female mice with intrasplenic transplants of testes, 12 testicular transplants were recovered after a period ranging from 23 to 224 days. In 2 testes that had been grafted for 23 and 38 days, spermatogenesis had not proceeded further than the primary spermatocyte stage. Normal numbers of the interstitial cells were noted in these 2 grafts. Histological features of the testes grafted for a period of over 150 days were generally similar to the corresponding transplants in the castrated male mice, except that the interstitial cells were perhaps more numerous (Fig. 6).

The uteri of the castrated female mice bearing intrasplenic testicular grafts were atrophic and weighed less than 25 mg. No x-zone was observed in the adrenal glands; androgenic effects were not noted in the kidneys and the submaxillary glands showed castration changes.

Discussion. In view of the relatively high percentage of successful transplants, the spleen appears to be a favorable site for experiments on the intraperitoneal transplantation of testes. Pfeiffer,³ and Turner¹³ reported that testicular transplants in the livers and kidneys of rats start to develop well but usually become infiltrated with fat and connective tissue and then degenerate.

Microscopical examination of the present material reveals that the intrasplenic transplants of testes in castrated male and female mice resemble, in general, the cryptorchid

⁹ Pfeiffer, C. A., Emmel, V., and Gardner, W. U., *J. Biol. and Med.*, 1940, **12**, 493.

¹⁰ Crabtree, C. E., *Endocrinology*, 1941, **29**, 197.

¹¹ Lacassagne, A., *C. R. Soc. Biol., Paris*, 1940, **133**, 180.

¹² Fekete, E., Chapter 3, *Biology of the Laboratory Mouse*, ed. by Snell, G. D., 1941.

¹³ Turner, C. D., *Am. J. Anat.*, 1938, **63**, 101.

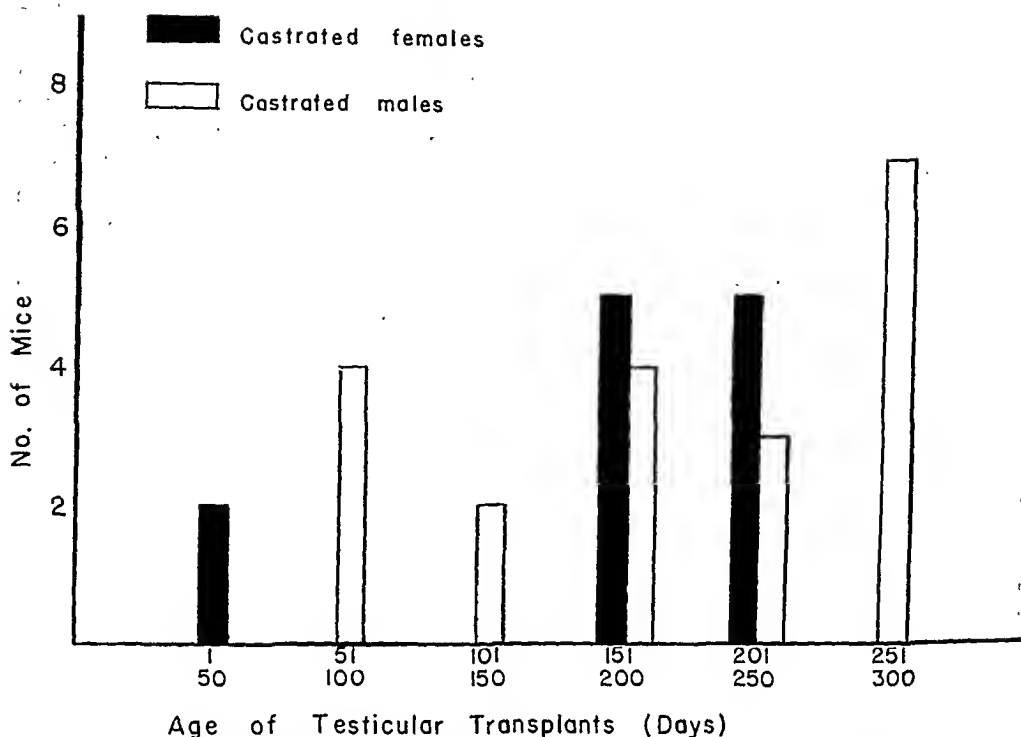


FIG. 1. Age of the intrasplenic testicular transplants in castrated male and female mice.

follows castration. A failure to obtain androgenic effects on the accessory genital organs of castrated male rats and rabbits occurred when androgens were administered so that they passed through the hepatic portal system before gaining access to the general circulation.³⁻⁷ Biskind and Biskind⁸ have reported that one granulosa cell-like tumor developed in an intrasplenic testicular graft in a castrated male rat. The fate of intrasplenic testicular transplants in castrated male and female mice and evidence for their endocrine effects are reported in this paper.

Materials and Methods. Inbred mice of

the A strain were used.[†] Both male and female mice were castrated when they were 1 to 3 months of age, and a testis obtained from 2- to 7-day-old male mice of the same strain was transplanted into the spleen of each animal at the time of castration. The animals were fed on a commercial diet (Nurishmix), with occasional supplements of grain (a mixture of wheat, oats, sunflower seeds, and calf-meal pellets), and water. The age of the intrasplenic transplants of testes at the time of autopsy is given in Fig. 1.

Results. The spleen provided a good vascular supply for the testicular grafts: they increased in size shortly after the transplantation as observed at laparotomy. The transplants usually protruded from the surface of the spleen; the largest measured about 4 x 8 mm in diameter (Fig. 2).

Twenty testicular grafts were recovered from castrated male mice bearing intrasplenic transplants for a period ranging from 63 to

³ Pfeiffer, C. A., *Am. J. Anat.*, 1936, **58**, 195.

⁴ Biskind, G. R., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 259.

⁵ Burrill, M. W., and Greene, R. R., *Proc. Soc. Exp. Biol. and Med.*, 1940, **44**, 273.

⁶ Burrill, M. W., and Greene, R. R., *Endocrinology*, 1942, **31**, 73.

⁷ Krichesky, B., Benjamin, J. A., and Slater, C., *Endocrinology*, 1943, **32**, 345.

⁸ Biskind, M. S., and Biskind, G. R., *Proc. Soc. Exp. Biol. and Med.*, 1945, **59**, 4.

[†] These mice were supplied by Doctors A. Gorman, C. W. Hooker, and L. C. Strong.

FIG. 5. Section of a testis grafted in the spleen of a castrated male mouse for 243 days showing spermatocytes, spermatids, and three spermiogenic cells (arrows pointed). Photomicrograph $\times 380$ (approx.).

FIG. 6. Section of a testis grafted in the spleen of a castrated female mouse for 165 days. Note the number of the interstitial cells and thin seminiferous epithelium. Photomicrograph $\times 86$ (approx.).

testes described in many other mammalian species.¹⁴ That the normal spermatogenic activity in retained testes is impaired by the higher temperature in the abdominal cavity is known, but information concerning the definite mechanisms that govern the normal process of spermatogenesis is still incomplete. The observation of large primary spermatocytes containing 2 or more nuclei in the testicular grafts indicates that the cytoplasmic divisions do not proceed normally even in the spermatogonia. It is interesting to find secondary spermatocytes, spermatids, and early formation of spermatozoa in a testis transplanted into the spleen for a period of 243 days. Complete spermatogenesis in testes that presumably resided inside the abdominal cavity has been reported in 2 hermaphroditic mice by Blotvogel,¹⁵ and Hooker and Strong.¹⁶

The maintenance of accessory genital organs in a castrated male mouse with vascularized adhesion of the testicular graft to the left kidney indicates that intrasplenic grafts produce androgen. The histological appearance of the interstitial cells of Leydig in the testicular transplants affords further indication of the functional activity. The castration effects found in organs of the castrated male mice bearing intrasplenic transplants of the testes suggest that the liver inactivates the androgenic hormone produced by the graft. With the mice thus physiologically castrated as far as androgen in the general circulation is concerned, it seems probable that the gonadotrophic hormones of the hypophysis are increased.¹⁷ This would

explain the functional appearance of the interstitial cells in the intrasplenic grafts and would agree with the observations of Nelson¹⁸ and Moore¹⁹ who reported that the hormone secretion by experimental cryptorchid testes in rats is stimulated by injections of gonadotrophic hormones. Turner¹³ observed hypertrophy and hyperplasia of the interstitial cells of Leydig in intraocular transplants of testes in castrated rats, but the transplants in noncastrated rats invariably contain sparse interstitial elements.

Histological study of the present material shows no indication that the intrasplenic testicular transplants in castrated male and female mice may develop into a granulosa cell-like tumor such as reported by Biskind and Biskind⁸ in a castrated male rat under similar circumstances. The failure to find tumors in the present experiment suggests that intrasplenic testicular transplants do not as readily become tumorous as do intrasplenic ovarian grafts in castrated mice.^{1,2}

Summary. Thirty-two successful testicular homotransplants were recovered from 40 castrated male and female mice of the A strain bearing the intrasplenic transplants for a period ranging from 23 to 275 days. The testicular transplants showed the general histological features of cryptorchid testes, although spermatids and sperm heads were noted in one case after 243 days of transplantation. The present material gave no indication of tumor development in the intrasplenic testicular transplants. The experimental data suggested that the endogenous androgen produced by the intrasplenic testicular transplant was inactivated when passing through the hepatic portal system.

¹⁴ Moore, C. R., Chapter 7, *Sex and Internal Secretions*, ed. by Allen, E., 1939.

¹⁵ Blotvogel, W., *Zentrabl. f. Gyn.*, 1932, 56, 2250.

¹⁶ Hooker, C. W., and Strong, L. C., *Yale J. Biol. and Med.*, 1944, 16, 341.

¹⁷ Burrows, H., *Biological Actions of Sex Hormones*, Cambridge Univ. Press, 1945, p. 45.

¹⁸ Nelson, W. O., *Proc. Soc. Exp. Biol. and Med.*, 1934, 31, 1192.

¹⁹ Moore, C. R., *Yale J. Biol. and Med.*, 1944, 17, 203.

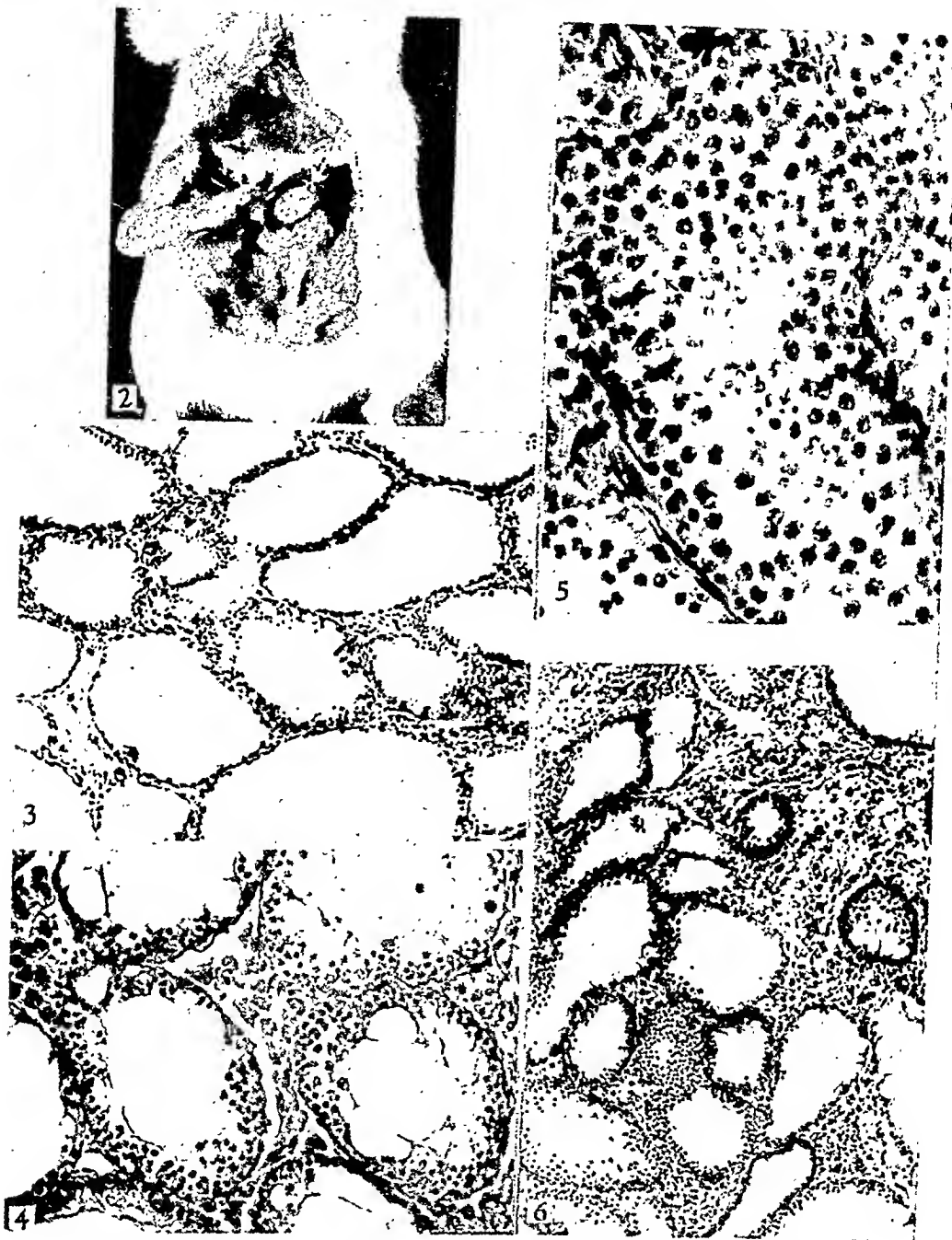


FIG. 2. Photograph of a castrated male mouse bearing an intrasplenic testicular graft for 274 days. Arrow indicates the graft. Note atrophy of the seminal vesicles and prostates.

FIG. 3. Section of a testis grafted in the spleen of a castrated male mouse for 257 days. Photomicrograph $\times 80$ (approx.).

FIG. 4. Section of a testis grafted in the spleen of a castrated male mouse for 208 days showing the interstitial cells and disorganized seminiferous epithelium. Photomicrograph $\times 192$ (approx.).

FIG. 5. Section of a testis grafted in the spleen of a castrated male mouse for 243 days showing spermatocytes, spermatids, and three spermiogenic cells (arrows pointed). Photomicrograph $\times 380$ (approx.).

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¹⁴ Moore, C. R., Chapter 7, *Sex and Internal Secretions*, ed. by Allen, E., 1939.

¹⁵ Blotvogel, W., *Zentrabl. f. Gyn.*, 1932, **56**, 2250.

¹⁶ Hooker, C. W., and Strong, L. C., *Yale J. Biol. and Med.*, 1944, **16**, 341.

¹⁷ Burrows, H., *Biological Actions of Sex Hormones*, Cambridge Univ. Press, 1945, p. 45.

¹⁸ Nelson, W. O., *Proc. Soc. Exp. Biol. and Med.*, 1934, **31**, 1192.

¹⁹ Moore, C. R., *Yale J. Biol. and Med.*, 1944, **17**, 203.

Toxicity Studies on Rutin.

ROBERT H. WILSON, T. G. MORTAROTTI, AND E. K. DOXTADER.

From the Pharmacology Division, Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture, Albany, California.

Rutin is a flavonol glycoside that has been found clinically to have a beneficial effect in a variety of conditions characterized by abnormal fragility of the capillaries.¹⁻⁴ Its action is thus similar to that of vitamin P, the term applied by Rusznyák and Szent-Györgyi⁵ to a preparation which they believed led to a decrease in capillary fragility. The substances which have been reported to possess this activity have been flavonol derivatives or compounds closely related to the flavonols,^{1,6} and of these rutin (quercetin rhamnoglucoside) is found in a number of common plants. The preparation of rutin from buckwheat is economically feasible.⁷

Toxicity data on the flavonol glycosides are scanty and knowledge of chronic toxicity is especially limited. Since clinical experience indicates that treatment, to be effective, must be continued for long periods, chronic toxicity studies are especially important. Garino⁸ administered rutin, quercitrin, hesperidin and naringin by mouth or parenterally into dogs; some of these compounds were eliminated in the urine unchanged, and none was found to be toxic. Czimmer⁹ reported, without details, that a quercetin glycoside

obtained from forsythia could be administered parenterally in relatively large doses for a long period of time to a variety of animals without eliciting any symptoms. (This glycoside from forsythia has been identified by Couch, Naghski and Porter¹⁰ as rutin). von Jeney, Vályi-Nagy, Vácz and Mihalik¹¹ injected 10 mg/kg of quercitrin into dogs without untoward effects, although blood lactic acid values were lowered. Wilson and DeEds¹² fed diets containing up to 1% of the flavonone glycosides, naringin and hesperidin, to rats for 200 days. Growth and organ weights of the animals were not affected and the tissues of hesperidin-treated animals, which were examined microscopically, were normal. Giltner¹³ has fed rutin to albino guinea pigs and exposed the animals to light; these animals did not show signs of light sensitization such as occurs in animals eating buckwheat, an important point, since the principal source of rutin at the present time is buckwheat. Clinical reports by Griffith, Couch, Lindauer² and by Shanno³ indicate that a daily oral intake of 40 to 120 mg of rutin for periods up to 9 months can be taken without the patients showing any signs of toxicity from the drug, and Griffith, Lindauer, Shanno and Couch, in their exhibit at the meeting of the American Medical Association in 1946, stated that in some cases the time had been extended to 36 months.

Experimental. Acute toxicity. In the

¹⁰ Couch, J. F., Naghski, J., and Porter, W. L., *J. Am. Chem. Soc.*, in press.

¹¹ v. Jeney, A., Vályi-Nagy, T., Vácz, L., and Mihalik, St., *Arch. exp. Path. Pharmacol.*, 1940, 194, 718.

¹² Wilson, R. H., and DeEds, F., *Food Research*, 1940, 5, 89.

¹³ Giltner, L. T., personal communication.

¹ Scarborough, H., *Biochem. J.*, 1945, 39, 271.

² Griffith, J. Q., Jr., Couch, J. F., and Lindauer, M. A., *Proc. Soc. Exp. Biol. and Med.*, 1944, 55, 228.

³ Shanno, R. L., *Am. J. Med. Sciences*, 1946, 211, 539.

⁴ Kushlan, S. D., *Gastroenterology*, 1946, 7, 199.

⁵ Rusznyák, St., and Szent-Györgyi, A., *Nature*, 1936, 138, 27.

⁶ Bruckner, V., and Szent-Györgyi, A., *Nature*, 1936, 138, 1057.

⁷ Couch, J. F., Naghski, J., and Krewson, C. F., *Science*, 1946, 103, 197.

⁸ Garino, M., *Z. physiol. Chem.*, 1913, 88, 1.

⁹ Czimmer, A. G., *Arch. exp. Path. Pharmacol.*, 1936, 183, 587.

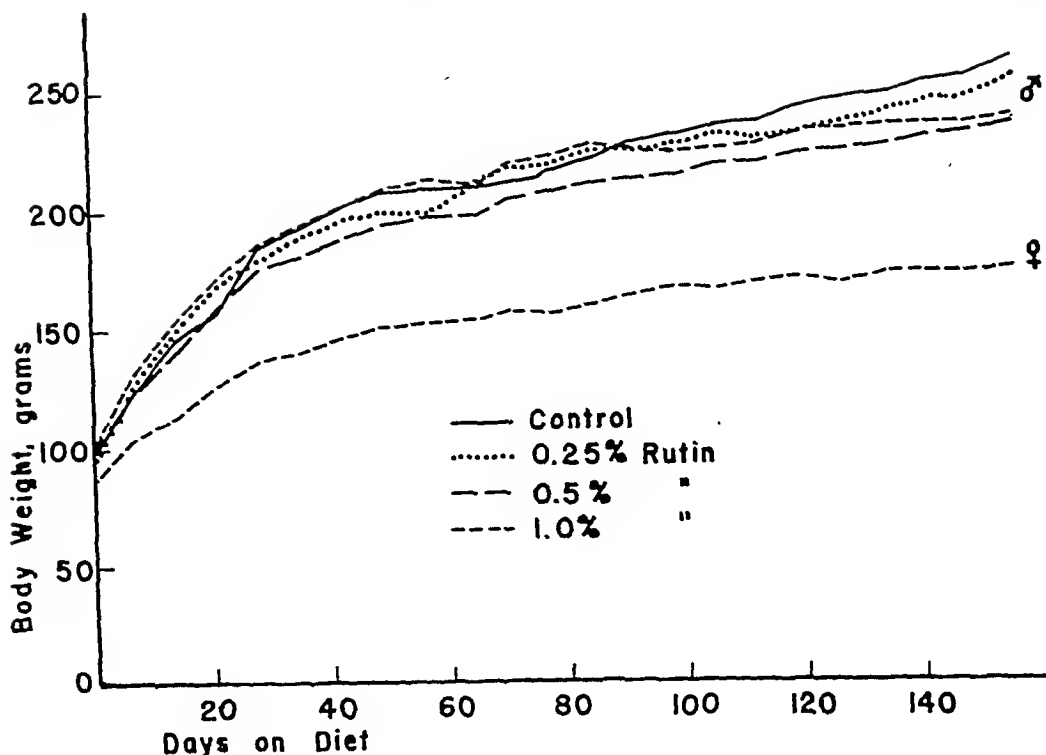


FIG. 1.
Growth curves of rats on Rutin-containing diets.

course of studies to be published later on the pharmacology of rutin,* certain data have accumulated on the acute toxicity of the substance. Adult rats and guinea pigs weighing 200 to 350 g have been injected with solutions containing 10 mg/ml of rutin. Ordinarily, the solvent was 5% ethyl alcohol, the rutin being soluble in the hot solvent and precipitating out slowly enough on cooling so that injections could be made. Occasionally, Ringer's solution was used as the solvent; rutin is fairly soluble in Ringer's, but a darkening of the solution indicated some chemical change and, for this reason, such a solution was seldom used. Sixteen rats were given 10 mg per animal, or approximately 50 mg/kg, intraperitoneally, and 8 guinea pigs 10 mg per animal, about 30 to 40 mg/kg, intraperitoneally or intravenously. Twelve rabbits were injected intravenously

with a 20% solution of rutin dissolved in propylene glycol; the dosage was 100 or 200 mg/kg. No symptoms were observed in any of these animals.

Chronic toxicity. Albino rats were used for the studies of chronic toxicity. These animals, mostly males and weighing about 90 g at the start of the experiment, were divided into groups of 6 animals each and fed diets containing from 0.25 to 1.0% of rutin thoroughly mixed into finely ground Purina Dog Chow.[†] Growth curves and food intakes were recorded for 150 days (Fig. 1). Growth was essentially normal at all levels of rutin. The female animals receiving the 1.0% diet had no controls. These animals were used later in connection with studies on reproduction. Their growth curve is in-

* The rutin was furnished us by the Eastern Regional Research Laboratory of the U. S. Department of Agriculture, Philadelphia.

[†] "Purina Dog Chow" is named as part of the specification of the exact experimental conditions, and it is not implied that this product is better than or inferior to any other commercially prepared dog feed.

cluded on the chart to indicate that administration of the diet was for the same period as for the males on the same diet. At the end of this period, the control rats and those on the 0.25 and 0.5% rutin diets were autopsied and the liver, spleen, kidneys, adrenals, testes and heart were weighed. The weights of the organs did not deviate from control values to a statistically significant degree, and the gross appearance of all tissues was normal.

The 6 males and 6 females on the 1% rutin diet were continued on the diet following completion of the growth records. They were mated, with results recorded below, and afterwards observed periodically until they had been eating the diet for 400 days. At autopsy, the gross appearance of the tissues was normal; organ weights were not obtained.

Tissues from all of the animals (both the 150-day and 400-day rats) were embedded in paraffin and sections were stained with hematoxylin and eosin. Histopathological study of these sections was made by Dr. A. J. Cox, Jr., Stanford University School of Medicine, San Francisco, who reported that there were no striking changes in any of the rats as compared with the controls. The animals which received 1% rutin in the diet for 400 days showed slight irregularity in vacuolation of the cortical cells of the adrenals, which may signify a little irregularity in lipid content of the cells. However, this was not pronounced and is of doubtful significance. In 3 of the 6 female rats which received the 1% rutin diet there was distinctly more brown granular pigment in the renal epithelial cells than in the controls. However, since all of the control animals were males, this observation cannot be interpreted. The male rats receiving the same amount of rutin did not show this change.

It was mentioned above that at the end of 150 days, the animals on the 1% rutin diet were used for observations on reproduction. There were 6 pairs, caged together for a month. From these unions there resulted 4 litters from 3 of the females (one of which must have been impregnated immediately after the birth of the first litter).

One of the 4 litters was born dead and the other 3 were small (3 to 5 per litter). Since the average for the colony is considerably better than this, the experiment was repeated with variations using more animals.

At 3 months of age, 30 healthy female rats and 15 males were placed on their respective diets—Purina Dog Chow containing 1% of rutin for the experimental animals, and the chow alone for the controls. Fifteen females and all of the males ate the rutin diet. After a month the animals were divided into 15 groups, each group containing a male, a female on the rutin diet, and a control female which was a litter-mate of the experimental female. To keep the experimental animals from eating control diet, and the control rats from ingesting rutin, food was withheld during the periods when the animals of a group were caged together. These periods were limited to about 7 hours, and there were 18 of them in the month of the experiment. Admittedly such excessive handling would not be conducive to good breeding, but chances for impregnation should be equal for control and experimental rats. As a result of these matings there were 4 litters born to the females eating the rutin diet, and 2 litters to control females. The litters varied considerably in size, mortality was not extreme. It is concluded that 1% of rutin in the diet did not limit the fertility of these animals.

Several of these litters were allowed to grow until weaning. The young of the mothers on the rutin diet were as large and active as those of the control females.

As a further check on the influence of rutin on reproduction, estrus studies were made for a 4-week period on 6 female rats eating a 1% rutin diet and 6 control females. The length of the estrous cycle was not modified by the ingestion of rutin.

Discussion. Clinical use of rutin, as noted earlier, can be expected to be for long periods of time, as present indications are that discontinuance leads to a recurrence of the original condition. For this reason, the most important part of a toxicity study of rutin must be the effect of chronic ingestion. In-

gestion by rats of a diet containing 1% of rutin for 400 days amounts, in the adult animal, to about 0.6 g/kg/day, and 400 days is well over one-third of a normal rat life. Such an intake is far in excess of any dosage likely to be administered for therapeutic purposes.

Conclusion and Summary. Rutin, a flavonol glycoside with vitamin P activity, was studied for signs of acute and chronic toxicity. In rats and guinea pigs, intravenous and intraperitoneal injections of 30 to 50 mg/kg, and intravenous injections in rabbits of 100 to 200 mg/kg, were innocuous.

The rate of growth of albino rats was not affected when the diet contained as much as 1% of rutin, and after 400 days on such a diet, histological examination of the tissues showed no evidence of injury which could be definitely related to rutin administration. Organ weights of the experimental animals were normal. The length of the estrous cycle was the same in rats eating a 1% rutin diet as in control animals, reproduction was as good, and the young appeared to be as healthy. On the basis of the criteria employed, rutin is nontoxic both acutely and chronically.

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Two Antibiotics (Lavendulin and Actinorubin) Produced by Two Strains of Actinomyces. III. Toxicity and Therapeutic Studies.*

HARRY E. MORTON. (Introduced by A. N. Richards.)

From the University of Pennsylvania, School of Medicine, Department of Bacteriology, Philadelphia, Pa.

Two new antibiotics, lavendulin and actinorubin, produced by 2 strains of Actinomyces isolated from soil have been described.^{1,2} The active substances have been purified and isolated and found to be of a basic nature.³ It is the purpose of this publication to describe the toxicity and therapeutic studies which were carried out in mice.

Materials. Test animal. White mice weighing 17-19 g were used throughout the experiments.

Antibiotics. Partially purified preparations² of lavendulin and actinorubin were used. The purest preparation of lavendulin used contained 4.5% ash. By the agar plate method of assay³ 0.65 μ g per ml of nutrient agar inhibited the growth of *Escherichia coli*. By the method of assay in which serial

dilutions of the material are made in nutrient broth,² 0.078 μ g per ml of broth inhibited the growth of *E. coli*, P216. The purest preparation of actinorubin used contained 2.8% ash. By the agar plate method of assay,³ 0.55 μ g per ml of nutrient agar inhibited the growth of *E. coli*. By the method of assay in which serial dilutions of the material are made in nutrient broth,² about 0.2 μ g per ml of broth inhibited the growth of *E. coli*. It was more difficult to get a reproducible end-point in the assay of actinorubin in broth than was the case with lavendulin.

Test organism for protection studies. *Klebsiella pneumoniae*, American Type Culture Collection No. 8050 was used. It was found to be of serological type A by the Quellung reaction.

* This project has been supported by the Smith, Kline and French Laboratories, Philadelphia, Pa.

¹ Kelner, A., Koeholaty, W., Junowicz-Koeholaty, R., and Morton, H. E., *J. Bact.*, 1946, **51**, 591.

² Kelner, A., and Morton, H. E., to be published.

³ Junowicz-Koeholaty, R., and Koeholaty, W., to be published.

Technics. Dilutions of the antibiotics were prepared in sterile distilled water.

For determining toxicity a given dose of the antibiotic was injected intraperitoneally into each of 10 mice. The mice were observed daily for 14 days.

In each protection test the virulence of the

culture was checked. One ml amounts of an 18-hour-old broth culture of *K. pneumoniae*, serially diluted in broth, were injected intraperitoneally into mice. Usually 5 to 10 mice were injected with each dilution of the culture. At the time each dilution of the culture was injected into mice, 1 ml portions were made into poured agar plates. After 48 hours of incubation at 37°C the colonies were counted. Five to 10 bacilli, as judged by the poured agar plate count, killed over 50% of the mice, whereas 40 to 50 organisms could be depended upon to kill all of the mice. The challenging dose of culture was between 100 and 1000 times the minimum amount of culture required to kill all of the mice. For determining the therapeutic effect of the antibiotic, 100 to 1000 lethal doses of *K. pneumoniae* were injected intraperitoneally into each mouse, followed in a few seconds by an intraperitoneal injection of the drug. Usually 10 mice were injected with each graded dose of the drug. The mice were kept under observation for 14 days. The heart's blood of some of the animals dying during the course of the experiment was streaked on agar plates to determine that death was due to infection with *K. pneumoniae*.

Results. The results of injecting mice with varying dilutions of lavendulin are summarized in Table I. Intraperitoneal injection of 0.5 mg was fatal to all of 10 mice. The

results of the therapeutic action of lavendulin in mice against at least 100 lethal doses of *K. pneumoniae* are summarized in Table II.

TABLE I.
Results of Injecting Mice with Varying Doses of Lavendulin.

No. of mice	Dose of lavendulin	Results
10	1 mg	1 died, < 46 hr 1 " 53 " 7 " < 70 " 1 " 70 "
10	0.75 "	1 " 50 " 6 " < 70 " 1 " < 72 " 2 " < 104 "
10	0.5 "	6 " < 70 " 2 " < 104 " 1 " < 105 " 1 " 125 "
10	0.25 "	1 " < 71 " 3 " < 104 " 1 " on 6th day 5 alive after 14 days*
10	0.1 "	10 " " 14 " †
10	75 µg	10 " " 14 " ‡
10	50 "	10 " " 14 " §

* Wt at time of injection, 17-18 g. Avg wt at end of exper., 16.9 g.

† Wt at time of injection, 17-18 g. Avg wt at end of exper., 19.3 g.

‡ Wt at time of injection, 17-18 g. Avg wt at end of exper., 20.6 g.

§ Wt at time of injection, 17-18 g. Avg wt at end of exper., 20.4 g.

TABLE II.
Summary of Tests Demonstrating the Protective Action of Lavendulin Against *K. pneumoniae* in White Mice.

Dose of culture, ml	diln	Dose of lavendulin	No. of mice injected	No. of mice dying	No. of mice surviving
1	10-8	0	5	3	2
1	10-7	0	5	5	0
1	10-6	0	5	5	0
1	10-5	0	5	5	0
1	10-5	200 µg	10	0	10
1	10-5	100 "	10	0	10
1	10-5	50 "	10	0	10
1	10-5	25 "	10	1*	9
1	10-5	10 "	10	2†	8
1	10-5	5 "	10	7‡	3

* Died on the 12th day of the experiment. No *K. pneumoniae* in culture from the heart's blood.

† Died on the 2nd and 3rd days of the experiment. One mouse autopsied and *K. pneumoniae* cultivated from the heart's blood.

‡ *K. pneumoniae* cultivated from the heart's blood of each of 3 mice autopsied.

TABLE III.

Results of Two Experiments in Which Mice Were Injected Intraperitoneally with Varying Doses of Actinorubin.

No. of mice	Dose of actinorubin	Results
	mg	
10	3.43	10 died (3rd and 4th days)
10	1.37	10 died (3rd, 4th, and 5th days)
10	1.0	9 died (4th, 5th, and 9th days)
		1 alive 14th day
10	0.68	5 died (5th, 6th, 7th, and 10th days)
		5 alive 14th day
9	0.34	9 alive 14th day
10	1	8 died on 5th day
		1 died on 7th day
		1 alive after 14 days*
10	0.75	3 died on 5th day
		7 alive after 14 days†
10	0.5	3 died (8 to 13 days)
		7 alive after 14 days‡
10	0.35	2 died, 3rd and 8th day
		8 alive after 14 days§
10	0.25	All alive after 14 days§

* Wt at time of injection, 18-19 g. Wt at end of exper., 15 g.

† Wt at time of injection, 18-19 g. Avg wt of 5 mice at end of exper., 15.1 g.

‡ Wt at time of injection, 17-18 g. Avg wt at end of exper., 16.8 g.

§ Wt at time of injection, 17-18 g. Avg wt at end of exper., 19.9 g.

All of 10 mice were protected from death due to the test organism by 25 μ g of lavendulin. The ratio of the therapeutic dose to toxic dose of lavendulin is of the order of 1:20. Calculating the LD₅₀ of lavendulin and that dose of lavendulin which protected 50% of the mice in the presence of more than 1000 LD₅₀ of culture by the procedure of Reed and Muench,⁴ the ratio of therapeutic dose to toxic dose is of the order of 1:27.

The results of injecting mice with varying dilutions of actinorubin are summarized in Table III. All of 10 mice were killed by

⁴ Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, 27, 493.

TABLE IV.

Summary of the Tests Demonstrating the Protective Action of Actinorubin Against *E. pneumoniae* in White Mice.

Date of test: April 13, 1946.

Dose of culture ml	diln	Dose of actinorubin	No. of mice injected	No. of mice dying	No. of mice surviving
1	10 ⁻⁹	0	5	1	4
1	10 ⁻⁸	0	5	5	0
1	10 ⁻⁷	0	5	5	0
1	10 ⁻⁶	0	5	3	2
1	10 ⁻⁵	0	5	5	0
1	10 ⁻⁶	340 μ g	5	0	5
1	10 ⁻⁶	137 "	5	0	5
1	10 ⁻⁶	68.5 "	5	0	5
1	10 ⁻⁶	34 "	5	0	5
1	10 ⁻⁶	13.7 "	5	0	5
1	10 ⁻⁶	6.85 "	5	1	4
1	10 ⁻⁶	3.4 "	5	2	3
Date of Test: April 24, 1946.					
1	10 ⁻⁹	0	10	1	9
1	10 ⁻⁸	0	10	7	3
0.5	10 ⁻⁷	0	10	9	1
1	10 ⁻⁷	0	10	10	0
1	10 ⁻⁶	0	10	10	0
1	10 ⁻⁵	0	10	10	0
1	10 ⁻⁵	40 μ g	10	3	7
1	10 ⁻⁵	30 "	10	5	5
1	10 ⁻⁵	20 "	10	6	4
1	10 ⁻⁵	14 "	10	6	4
1	10 ⁻⁵	10 "	10	7	3
1	10 ⁻⁵	5 "	10	10	0
1	10 ⁻⁵	2.5 "	10	9	1

culture was checked. One ml amounts of an 18-hour-old broth culture of *K. pneumoniae*, serially diluted in broth, were injected intraperitoneally into mice. Usually 5 to 10 mice were injected with each dilution of the culture. At the time each dilution of the culture was injected into mice, 1 ml portions were made into poured agar plates. After 48 hours of incubation at 37°C the colonies were counted. Five to 10 bacilli, as judged by the poured agar plate count, killed over 50% of the mice, whereas 40 to 50 organisms could be depended upon to kill all of the mice. The challenging dose of culture was between 100 and 1000 times the minimum amount of culture required to kill all of the mice. For determining the therapeutic effect of the antibiotic, 100 to 1000 lethal doses of *K. pneumoniae* were injected intraperitoneally into each mouse, followed in a few seconds by an intraperitoneal injection of the drug. Usually 10 mice were injected with each graded dose of the drug. The mice were kept under observation for 14 days. The heart's blood of some of the animals dying during the course of the experiment was streaked on agar plates to determine that death was due to infection with *K. pneumoniae*.

Results. The results of injecting mice with varying dilutions of lavendulin are summarized in Table I. Intraperitoneal injection of 0.5 mg was fatal to all of 10 mice. The

results of the therapeutic action of lavendulin in mice against at least 100 lethal doses of *K. pneumoniae* are summarized in Table II.

TABLE I.
Results of Injecting Mice with Varying Doses of Lavendulin.

No. of mice	Dose of lavendulin	Results
10	1 mg	1 died, < 46 hr 1 " 53 " 7 " < 70 " 1 " 70 "
10	0.75 "	1 " 50 " 6 " < 70 " 1 " < 72 " 2 " < 104 "
10	0.5 "	6 " < 70 " 2 " < 104 " 1 " < 105 " 1 " 125 "
10	0.25 "	1 " < 71 " 3 " < 104 " 1 " on 6th day 5 alive after 14 days*
10	0.1 "	10 " " 14 " †
10	75 µg	10 " " 14 " †
10	50 "	10 " " 14 " ‡

* Wt at time of injection, 17-18 g. Avg wt at end of exper., 16.9 g.

† Wt at time of injection, 17-18 g. Avg wt at end of exper., 19.3 g.

‡ Wt at time of injection, 17-18 g. Avg wt at end of exper., 20.6 g.

§ Wt at time of injection, 17-18 g. Avg wt at end of exper., 20.4 g.

TABLE II.
Summary of Tests Demonstrating the Protective Action of Lavendulin Against *K. pneumoniae* in White Mice.

Dose of culture, ml	diln	Dose of lavendulin	No. of mice injected	No. of mice dying	No. of mice surviving
1	10 ⁻⁸	0	5	3	2
1	10 ⁻⁷	0	5	5	0
1	10 ⁻⁶	0	5	5	0
1	10 ⁻⁵	0	5	5	0
1	10 ⁻⁵	200 µg	10	0	10
1	10 ⁻⁵	100 "	10	0	10
1	10 ⁻⁵	50 "	10	0	10
1	10 ⁻⁵	25 "	10	1*	9
1	10 ⁻⁵	10 "	10	2†	8
1	10 ⁻⁵	5 "	10	7‡	3

* Died on the 12th day of the experiment. No *K. pneumoniae* in culture from the heart's blood.

† Died on the 2nd and 3rd days of the experiment. One mouse autopsied and *K. pneumoniae* cultivated from the heart's blood.

‡ *K. pneumoniae* cultivated from the heart's blood of each of 3 mice autopsied.

mentally normal. The spleens were reduced in size and the capsule was thickened by fibrous tissue growth.

These observations indicated that the liver was extensively damaged by the substances injected but regenerated more or less completely by the end of 2 weeks. Actinorubin also caused tubular damage in the kidneys and largely destroyed the lymphoid elements of the thymus. Lymphoid tissues of the spleen did not appear to have been damaged. Fibrosis of the splenic capsule may be attributed to the intraperitoneal injection of the material. Although the mice which received actinorubin showed the more extensive pathological changes, it must be borne in mind that, on a weight basis, these mice received three times more drug than the mice which received lavendulin.

Summary. The toxic doses (LD_{100}) of actinorubin and lavendulin have been determined for white mice by intraperitoneal injection. In the case of lavendulin it was 0.5 mg, while in the case of actinorubin it was 1.37 mg. The ability of the antibiotics to protect mice from death following the intraperitoneal injection of 100 to 1000 M.L.D. of *K. pneumoniae* was determined. In the case of lavendulin this therapeutic dose was 25 μ g and in the case of actinorubin it was found to be 13.7 μ g in one experiment. In a second experiment 0.68 mg of actinorubin killed 50% of the mice while 30 μ g protected 50% of the mice against 100 lethal doses of *K. pneumoniae*. Further testing of the 2 antibiotics has not been possible due to the limited supplies of the substances.

15783

Production of Increased Capillary Fragility in Rats Following Irradiation.*

J. Q. GRIFFITH, JR.,[†] ELEANOR ANTHONY, E. P. PENDERGRASS, AND R. PERRYMAN.

From the Robinette Foundation, the Medical Clinic and the Department of Radiology of the Hospital of the University of Pennsylvania.

In the course of a clinical study of increased capillary fragility, it became desirable to develop a corresponding study in an experimental animal. A method for measuring the increase in fragility was needed as well as a means for producing increased fragility. Hundreds of unsuccessful experiments were conducted in rats and guinea pigs before the problem was satisfactorily solved. Only the successful method, however, will be described.

Method for measuring capillary fragility in rats. Under general anesthesia the peri-

toneal cavity is opened by a long midline longitudinal incision, care being taken to exert no traction on the peritoneum. The abdominal wall is carefully folded back until an artery and vein are disclosed. Then, under a good light, the peritoneum is grasped on each side of the vessels and stretched between the fingers so that the lumen of the vein is obliterated but the artery can still be seen pulsating. The tension is maintained for 2 minutes after which it is relaxed. The peritoneal area supplied by the vessels is carefully inspected and, if the fragility is increased, one or more petechia will appear distal to the point of venous obstruction, usually entirely separate from any visible vessel. Actually, in a positive case, 6 or 8 such hemorrhages usually occur. If desired, the process can be repeated in the same animal, using other vessels. No petechial hemorrhages occur in the normal animal.

* The work described in this paper was done in part under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Trustees of the University of Pennsylvania. More recently it has been continued as a project of the Army Research and Development Board.

[†] Atwater Kent Fellow in Medicine.

an intraperitoneal injection of 1.37 mg. The results of one test (on April 13) on the therapeutic action of actinorubin in mice against 100 to 1000 lethal doses of *K. pneumoniae* are summarized in Table IV. All of 5 mice were protected from death by 13.7 μ g of actinorubin. The ratio of the therapeutic dose to the toxic dose of actinorubin in this experiment was of the order of 1:100. No explanation is offered of the fact that 2 out of the 3 mice, which received a dose of culture estimated to be in excess of 100 lethal doses of *K. pneumoniae*, failed to die.

Repeating the experiment in part, using 100 lethal doses of *K. pneumoniae*, gave less protection than in the previous test. Comparing the dose of 30 μ g of actinorubin which protected 50% of the mice against 100 lethal doses of culture with 680 μ g which killed 50% of the animals gives a ratio of 1:22. Practically the same ratio was obtained using LD₅₀ as calculated by the method of Reed and Muench.⁴ The effects of aging and repeated freezing and thawing of the solutions of actinorubin were not determined.

Since actinorubin appeared more active *in vivo* against *K. pneumoniae* than lavendulin, 2 preliminary tests were made to determine rate of absorption following intraperitoneal or oral administration. Each of 8 mice were injected intraperitoneally with 1.3 mg of actinorubin. This would ordinarily inhibit the growth of *E. coli* in Bacto-nutrient broth, pH 7.3, in a dilution of about 1:6875. After varying intervals of time one of the mice was anesthetized with diethyl ether and bled from the axilla according to the technic described by Kuhn.⁵ Fifteen minutes after the intraperitoneal injection, the blood of a mouse inhibited *E. coli* in a dilution of 1:320; after 30 minutes, the blood of another mouse inhibited in a dilution of 1:160. After 75 minutes the blood of another mouse inhibited the test organism in a dilution of 1:10. No actinorubin was detected in a 1:10 dilution of blood samples taken from mice at 2-, 3-, 4-, 5- and 6-hour intervals.

Seven mice were each given 3.43 mg of actinorubin in 0.25 ml by stomach tube. No actinorubin was detectable in the blood when tested after $\frac{1}{4}$, $\frac{1}{2}$, 1, 2, 3, 4, and 5 hours, one animal being sacrificed at each period.

Pathological Changes.[†] Mice which survived the larger test injections of lavendulin and actinorubin (Tables I and III) failed to gain weight or even lost weight during the 2 weeks of observations. Postmortem examination of 6 mice 16 days after the intraperitoneal injection of each with 0.75 mg of actinorubin revealed the following abnormalities: Body weights varied from 13.4 to 19.7 g (normal weight for the same lot and age was 31 g). The livers were irregularly distorted with a reduction in the number of lobes or fusion of the lobes to form a rather solid mass which often completely enclosed the gall bladder. Microscopic examination revealed eosinophilic, necrotic foci in some livers of the animals but in all fibrous tissue stroma was irregularly increased and the lobules often were more or less distorted. A true cirrhosis was not developed, however. The kidneys were enlarged and of a pale, dull brown color. In all of the animals the convoluted tubules were dilated and lined by flattened epithelial cells, whereas the glomeruli were lined by columnar cells. The dilated tubules in 4 animals contained dense eosinophilic casts. In each of the 6 animals the thymus was reduced more or less but in 4 of the animals little remained but reticulum and epithelial elements. The other viscera were unchanged.

Five mice which survived 0.25 mg of lavendulin were sacrificed 15 days after the intraperitoneal injection of the drug. Body weights varied from 12.9 to 19.9 g (normal weight for the same lot and age was 31 g). The livers were distorted to a lesser degree than in the group which received actinorubin although the abnormalities were of the same type. The kidneys were unchanged. The thymus in 3 of the animals appeared reduced in size but microscopic sections were es-

[†] We are indebted to Dr. Herbert Ratcliffe, Department of Pathology, University of Pennsylvania, School of Medicine, for the examination of some of the test animals.

⁵ Kuhn, L. R., *Science*, 1946, 93, 504.

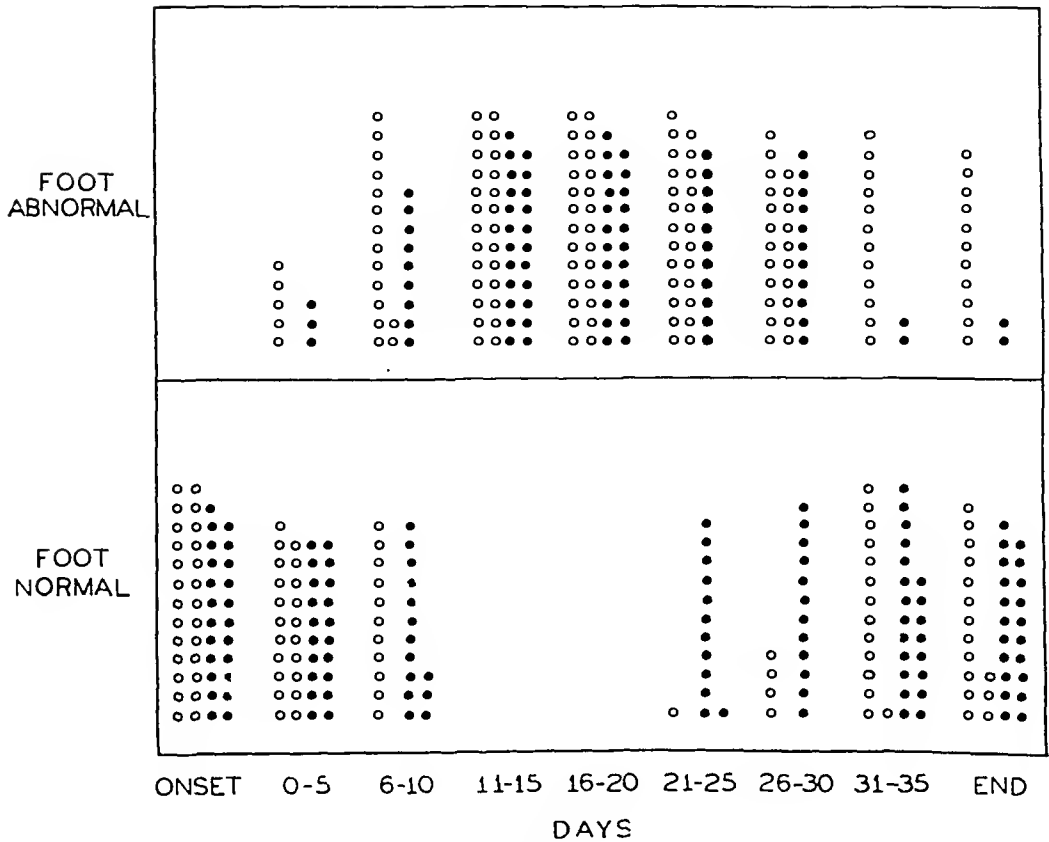


FIG. 1.

All rats were given irradiation to one foot, 2385 r in a single exposure. Closed circles represent animals treated with rutin, while open circles represent untreated animals. For other details, see text.

difference between the treated and untreated groups. There is no significant difference between the 2 groups in the time of onset of the reaction, averaging the 10th day for the control group and the 11th day for the rutin-treated group (Fig. 1). In this chart each animal is listed once for each 4-day period, appearing in the lower rectangle if the treated and untreated foot were indistinguishable, and in the upper if a definite reaction were present. It is apparent that,

from the 21st to the 25th day 12 of the animals receiving rutin returned to normal and only one of the control group. At the time of termination of the experiment, after the 35th day, only 2 of the treated animals still showed an abnormal foot, while 11 of the control group were still abnormal.

Conclusion. Under the conditions of the experiment, rutin appeared to hasten the recovery time after irradiation.

Method for producing increased capillary fragility in rats. A substance capable of emitting alpha radiation is introduced into the peritoneal cavity of normal rats. The substance used was commercial radon ointment, 500 E.S.U., (200 microcuries 1 cc)† prepared in either lanolin (15 animals) or olive oil (5 animals). Radon ointment contains small amounts of radon gas. Ninety per cent of the radiation is composed of alpha particles while the remainder consists of beta and gamma particles. Amounts used were 0.5 cc (2 animals), 1.0 cc (16 animals) and 1.5 cc (2 animals). When the

olive oil preparation was used, the material was simply injected through a needle, while with the lanolin a small incision had to be made. No attempt was made to remove any of the material subsequently. Fragility was tested 1 to 8 weeks after the peritoneal injection. In every animal fragility was markedly increased.

Controls consisted of 5 animals injected with lanolin, 5 injected with olive oil, and 10 entirely untreated animals. None showed any petechia during the test.

Conclusion. Exposure of the peritoneal cavity of the rat to irradiation in excessive amount will produce an increase in capillary fragility.

† Dosage as estimated by manufacturer.

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Effect of Rutin on Recovery Time from Radiation Injury in Rats.*

J. Q. GRIFFITH, JR.,† ELEANOR ANTHONY, E. P. PENDERGRASS, AND R. PERRYMAN.

From the Robinette Foundation, the Medical Clinic and the Department of Radiology of the Hospital of the University of Pennsylvania.

Rutin, a flavonol glucoside derived from various plants including buckwheat, has been shown by Griffith, Lindauer and Couch¹ to decrease capillary fragility in man, when that fragility was originally increased. One of the effects of excessive radiation appears to be an increase in capillary fragility.² There-

fore, an experiment was planned to discover whether rutin might have a favorable effect on radiation reaction.

Method. All animals were given radiation to one leg, 2385 r, in a single dose, using 200 KV, 15 ma., ½ mm cu. + 1 mm al filter, 26 cm skin target distance. At the same time half the animals were given a pellet containing 20 mg of rutin, implanted along the lateral aspect of the abdominal wall, and this was repeated thereafter every third day, for a period of 36 days. The remaining animals were not given rutin. At the end of the experimental period 23 rutin-treated animals and 26 untreated controls survived. Animals were examined every fourth day and the extent of the reaction noted. The observed reaction consisted of an erythema, a moderate swelling and, in some cases, ulceration.

Results. The extent of the initial reaction is not charted, as there was no significant

* The work described in this paper was done in part under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Trustees of the University of Pennsylvania. More recently it has been continued as a project of the Army Research and Development Board. Abbott Laboratories, Chicago, Ill., provided the Rutin, and, by a grant, aided the study.

† Atwater Kent Fellow in Medicine.

¹ Griffith, J. Q., Jr., Couch, J. F., and Lindauer, M. A., *Proc. Soc. Exp. Biol. and Med.*, 1944, 55, 228.

² Griffith, J. Q., Jr., Anthony, E. M., Pendergrass, E. P., and Perryman, R., *Proc. Soc. Exp. Biol. and Med.*, 1947, 64, 331.

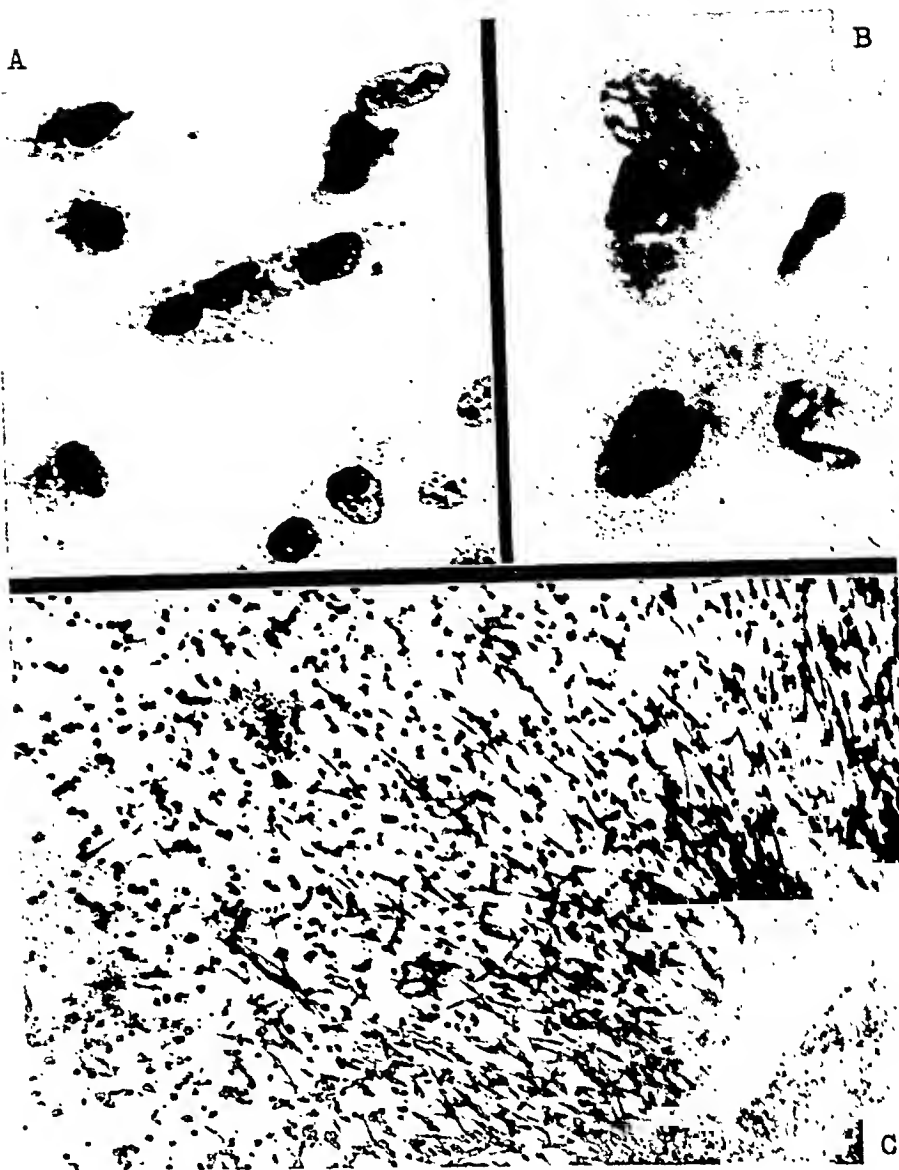


FIG. 1.

Detached and rounded fibroblasts in cultures treated with 10^{-5} M colchicine solution for periods from 15 minutes to 1 hour. Giemsa's stain. A, $\times 800$. B, $\times 1800$. C, $\times 120$.

the resting cell.

As far as can be judged from the illustrations in the paper of Gavrilov and von Bistram,⁴ it is possible that these authors

⁴ Gavrilov, W., and von Bistram, D., *Bull. Assn. franc. p. l'étude du cancer*, 1939, **28**, 319.

⁵ Ludford, R. J., *Arch. f. exp. Zellforsch.*, 1936, **18**, 411.

have observed in tissue cultures treated with colchicine, the phenomenon described by us. Ludford⁵ has noted that resting cells under the influence of weak solutions of colchicine tend to become less spread out and more rounded.

Summary. Chick embryo heart fibroblasts cultured *in vitro*, when immersed in colchicine

Effect of Colchicine on Resting Cells in Tissue Cultures.

B. MISZURSKI AND L. DOLJANSKI. (Introduced by L. Olitzki.)

From the Department of Experimental Pathology (Cancer Laboratories), The Hebrew University, Jerusalem, Palestine.

During our investigations on the effects of colchicine on cells growing *in vitro* we found that this drug, in addition to its action on the division mechanism of cells, also has a definite effect on the cytoplasm of resting cells.

The design of the experiments was as follows: Forty-eight-hour-old hanging drop cultures of chick embryo heart fibroblasts were immersed in a 10^{-4} or 10^{-5} M solution of colchicine in Ringer. The effect of colchicine was studied from the start of the experiment up to a period of 8 hours in the living state on the warm stage, and in preparations fixed in Carnoy's fluid and stained *in toto* with Giemsa's stain. In another series of experiments the colonies of fibroblasts were cultured for 24-48 hours in media containing similar concentrations of colchicine and were examined in the living state, as well as after fixation and staining.

In a culture of fibroblasts immersed in a colchicine solution of one of the above concentrations, it can be ascertained that, after 5-15 minutes the surfaces of the individual cells forming the zone of outgrowth display an extraordinary activity. The cell processes which connect neighboring cells or extend into the medium are withdrawn and the cells become independent of each other. Such a detached cell sends out pseudopodia in all directions; these pseudopodia are in constant motion, being rapidly projected and retracted. This motion is so rapid and so violent, that the peripheral portions of pseudopodia are often torn off. The cells tend to assume a polygonal or spherical shape and their cytoplasm becomes increasingly basophilic. The superficial cell layers remain active for many hours, short, broad, bleb-like pseudopodia, appearing and disappearing with great rapidity from all sides of the cell body. This picture closely resembles the behavior of the

cytoplasm of dividing cells during anaphase and telophase. Often the cells become detached not singly, but as small groups of 2, 3 or more. They remain together as a small clump and this unit shows at its periphery all the phenomena described for the surface of the individual cells (Fig. 1, A and B).

The cell colony treated with colchicine soon loses its regular texture and is composed not of radially arranged spindle cells but of separated single cells and small cell conglomerates (Fig. 1, C). The process of disruption of the texture of the culture is initiated at the periphery and spreads towards the center. Usually within an hour the whole cell colony looks as if it were torn to shreds.

Most of the loosened and rounded fibroblasts do not show any signs of degeneration during the period of observation and the structure of the resting nuclei remains completely unaltered. Only occasionally there is a moderate hydropic vacuolization of the cytoplasm. Even prolonged treatment with colchicine in the above mentioned concentrations has no lethal effect on the cells. Cell colonies cultured for 48 hours in medium containing a colchicine solution 10^{-5} M show an abundant outgrowth after being transferred to normal medium.

The view has often been expressed¹⁻³ that the essential point in the action of colchicine on the mitotic process is the suppression of the gelation of the spindle. Our observations suggest that colchicine is able to affect not only the spindle substance but also the sol-gel equilibrium of the cortical layer in

¹ Beams, H. W., and Evans, T. C., *Biol. Bull.*, 1940, **79**, 188.

² Ludford, R. J., *J. Nat. Canc. Inst.*, 1945, **6**, 89.

³ Wilbur, K. M., *Proc. Soc. Exp. Biol. and Med.*, 1940, **45**, 696.

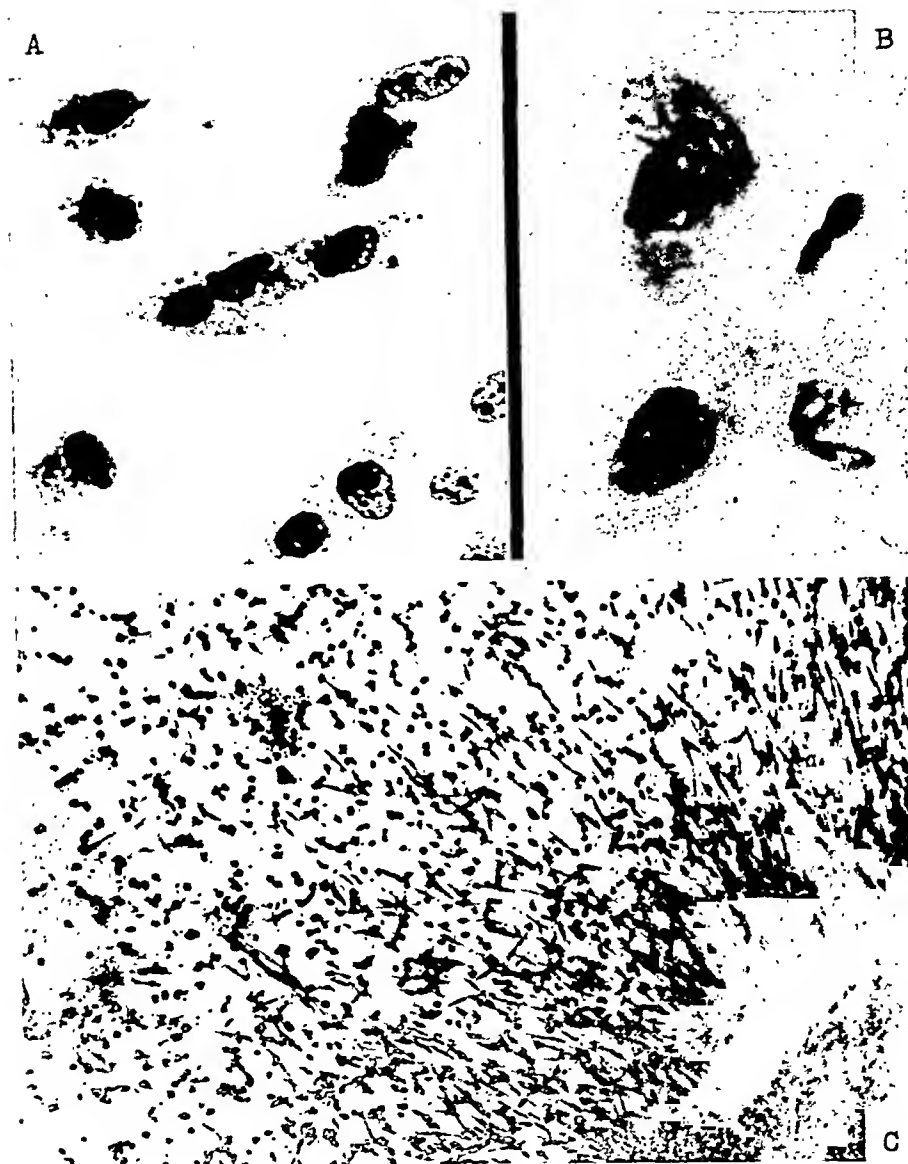


FIG. 1.

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have observed in tissue cultures treated with colchicine. the phenomenon described by us. Ludford⁵ has noted that resting cells under the influence of weak solutions of colchicine tend to become less spread out and more rounded.

Summary. Chick embryo heart fibroblasts cultured *in vitro*, when immersed in colchicine

solutions 10^{-4} or 10^{-5} M, show a tendency to assume a spherical shape. Their surfaces display a considerable activity as manifested by rapid protrusion and withdrawal of pseudopodia. Subsequently the zone of out-

growth of the culture splits up into detached elements, comprising one or more cells. This whole process is not lethal to the cells and is, to a great extent, reversible.

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Production of Polycythemia in Rabbits by Anoxia and Cobalt.*

JOSEPH GOODMAN (with the technical assistance of Byron Howells.)
(Introduced by D. R. Drury.)

From the Department of Aviation Medicine, University of Southern California, Los Angeles, Calif.

A need for polycythemic rabbits as experimental animals led to the consideration of methods of producing the state of polycythemia in this species. There are at least 2 experimental methods for inducing this condition. The older and better known of these is the response to anoxia induced by high altitude; another is the administration of cobalt salts.

Although the stimulating effect of lowered oxygen tension is well known and has been extensively studied, its mechanism is not as yet clearly understood. The effect has been noted in many animals other than man.¹ These include monkeys,² dogs,³ rats³ and rabbits.³⁻⁵

Armstrong and Heim^{4,5} reported on the exposure of young male rabbits for 4 hours daily 5 days a week to pressures equivalent to 18,000 feet. In addition to this treatment the animals were subjected to an alti-

tude tolerance test on the 7th day. This tolerance test involved rapid decompression to the point where the animals became unconscious. The equivalent altitude attained was noted and the animal immediately returned to ground level. In the 3rd week of their experiment a deterioration set in and the hemoglobin and hematocrit levels depreciated to lower than the original values. They concluded that rabbits were quite unsatisfactory for this work.

Later Thorn, Jones, Lewis, Mitchell and Koepf³ conducted a series of experiments in which they exposed rabbits to pressures equivalent to 18,000 and 25,000 feet for 4 hours a day 7 days a week. The 18,000-foot animals were continued for 5 weeks whereas the 25,000-foot experiment was discontinued at the end of 3 weeks because of excessive fatalities. Those exposed to 18,000 feet showed a temporary delay in growth, together with a slight drop in the concentration of serum, sodium chloride and CO₂ combining power and a decrease in the weight of the thymus gland. These changes were accompanied by an increase in O₂ capacity of the blood, in the hematocrit values and in the adrenal gland weight. Seventy-five percent of those exposed to 25,000 feet died of hemorrhage into the lungs and/or herniation of distended loops of the intestines into the thoracic cavity. There were no fatalities in the 18,000-foot group, nor was there evi-

* This work was supported in part by the OSRD and in part by the U. S. Army Air Force, Air Materiel Command, Wright Field, Dayton, Ohio.

¹ McFarland, R. A., *J. Comp. Physiol.*, 1937, **23**, 244.

² Jasper, H. H., *Canadian National Research Council, Ottawa*, 1942.

³ Thorn, G. W., Jones, B. F., Lewis, R. A., Mitchell, E. R., and Koepf, G. F., *Am. J. Physiol.*, 1942, **137**, 606.

⁴ Armstrong, H. G., and Heim, J. W., *J. Aviation Med.*, 1938, **9**, 45.

⁵ *Ibid.*, 1938, **9**, 92.

dence of degeneration in the hemopoietic system.

It may be noted that little is known of the mechanism or the optimum conditions for producing polycythemia by this method.

Another technic for producing polycythemia, whose mechanism is however even less clearly understood, was reported by Waltner and Waltner.⁶ They induced polycythemia by the administration of cobalt. This method has been used on several species but was found to be slow and sometimes accompanied by toxic symptoms. Frost *et al.*⁷ produced a temporary polycythemia in adult dogs by including cobalt in the food. However, they found this toxic to young growing dogs. On the other hand Stanley, Hopps and Hellbaum⁸ reported the development of polycythemia in rats by a subcutaneous injection without unfavorable symptoms.

Methods. It was decided to study the possibilities of both the above mentioned technics for producing experimental polycythemia. The routine of the cobalt injections was patterned after that employed by Stanley *et al.*⁸ The exposure time at altitude was materially increased above the time used by previous workers, but remained intermittent. This allowed the animals some time for recuperation under more nearly optimum living conditions at sea level. Altitude and cobalt stimuli were tried separately and together on immature male rabbits. Twelve animals, averaging 3½ lb. were divided into 4 groups of 3 rabbits each. Group I served as controls remaining at sea level pressures and without cobalt injections. Group II was subjected to an ambient pressure equivalent to an altitude of 20,000 feet, 16 hours a day, 5 days a week. Group III was injected subcutaneously with 1 ml of sterile cobalt solution per day 6 days a week, but remained at ground level (300 feet above sea level).

Group IV was injected subcutaneously with 1 ml of cobalt solution per day 6 days a week and was subjected to a pressure equivalent to 20,000-foot altitude 16 hours a day 5 days a week. The cobalt solution consisted of 10 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ per cc made up to isotonicity with NaCl. This was injected subcutaneously into a shaved area of the back and shoulders. The altitude was simulated in a decompression chamber. The altitude animals rested at ground levels (300 feet) each day for 8 hours and over Saturday and Sunday night, a period of 56 hours. The altitude "flights" were continued for 10 weeks. This was followed by a 2½-week ground level observation period.

The animals were weighed and sampled twice a week, on Mondays at the end of the 56-hour sea level resting period and on Thursdays after 3 of the 5 successive night flights of 16 hours. The blood samples were taken from the marginal ear vein. Hemoglobin was determined by the acid hematin method with a Klett Summerson colorimeter. Hematocrit values were measured in Wintrobe tubes. Plasma volume was determined by the dye method using T-1824.⁹ The plasma volume was measured only 4 times during the course of the entire experiment. These were, first during the prestimulus period, next after 3 weeks of exposure to altitude and/or cobalt, then after 10 weeks of stimuli and lastly at the end of the poststimuli observation period. The dye determination technic consisted of the injection of a weighed amount of a 1% dye solution in isotonic saline. After 8 minutes a blood sample was taken, using 1.6% sodium oxalate solution as an anticoagulant. The blood sample-oxalate ratio was determined in each case by weighing and the ratio used to correct the readings obtained. The intensity of the blue color in the plasma was measured on a Beckman quartz spectrophotometer, using the 620 μ absorption band.

Results. The groups of animals employed were too small to permit satisfactory statistical analysis. However, the response to the

⁶ Waltner, K., and Waltner, K., *Klin. Wochenschr.*, 1929, 8, 313.

⁷ Frost, D. V., Spitzen, E. H., Elvehjem, C. A., and Hart, E. B., *Am. J. Physiol.*, 1941, 134, 746.

⁸ Stanley, A. J., Hopps, H. C. and Hellbaum, A. A., *Proc. Soc. Exp. Biol. and Med.*, 1946, 61, 130.

⁹ Price, Phillip B., and Longmeir, William P., *Johns Hopkins Hosp. Bull.*, 1942, 71, 51.

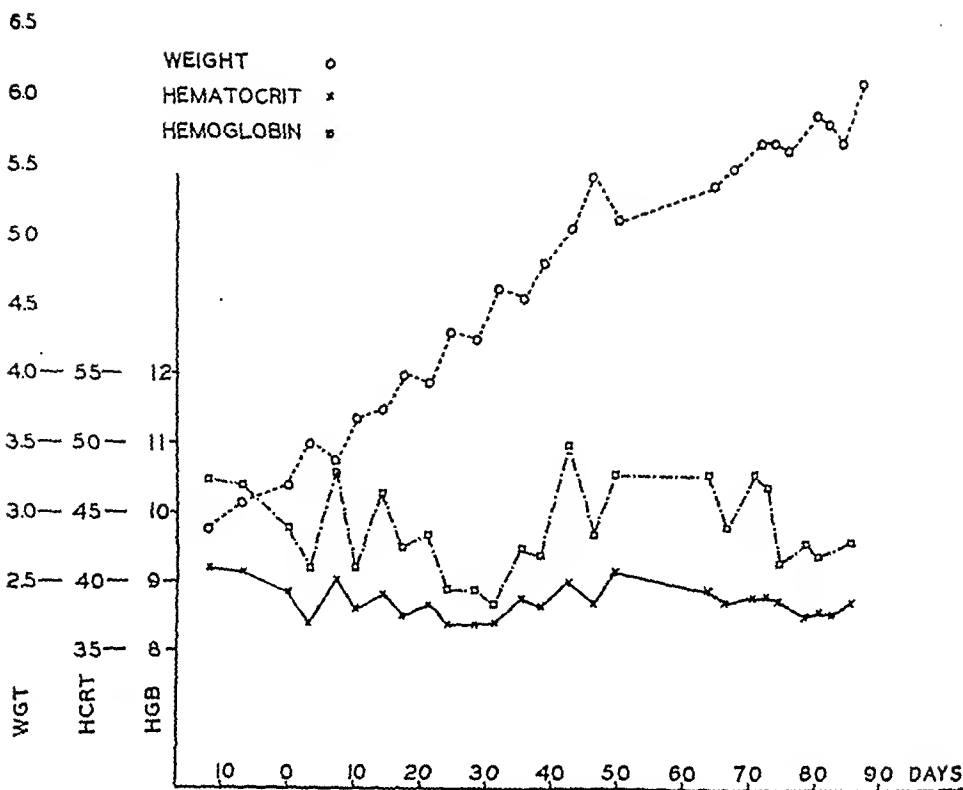


FIGURE 1 SEA LEVEL CONTROL
Weight, hematocrit, and hemoglobin averages of the control rabbits.

stimuli was definite and conclusively demonstrates the possibility of producing polycythemia in this species without deterioration.

Fig. 1 shows the average of the weight, hemoglobin and hematocrit values of the control group. (Only 2 animals are reported in this group. One was stolen on the second week of the experiment.)

The response to altitude alone is shown in Fig. 2. Each parameter represents the average value for 3 animals. (No fatalities). The exposure to altitude prompted a decrease in weight, followed by a period of slow recovery with a final resumption of a growth rate equivalent to that of the controls. Hemoglobin and hematocrit values both showed a rapid increase during the weekend resting period after the first exposures to altitude. This sloped off; first into a period of little change and then into a gradual climb which

persisted as long as the stimulus was applied. The decrease in hemoglobin and hematocrit from 25th to 35th day coincided with a similar depression in the control group and a period of high summer heat. It is to be noted that, during the control period following the altitude exposure the hemoglobin and hematocrit values returned rapidly toward the normal levels.

One of the cobalt-injected, ground level group, died apparently from the toxic effects of the cobalt combined with severe anorexia. The data in Fig. 3 represent the average of the values obtained from the 2 remaining animals. The weight curve generally follows that of the controls. The hemoglobin value dropped slightly when the injection started, followed by a gradual increase for the period of the injections, but did not attain values comparable to those in the animals subjected to altitude. The return to normal after in-

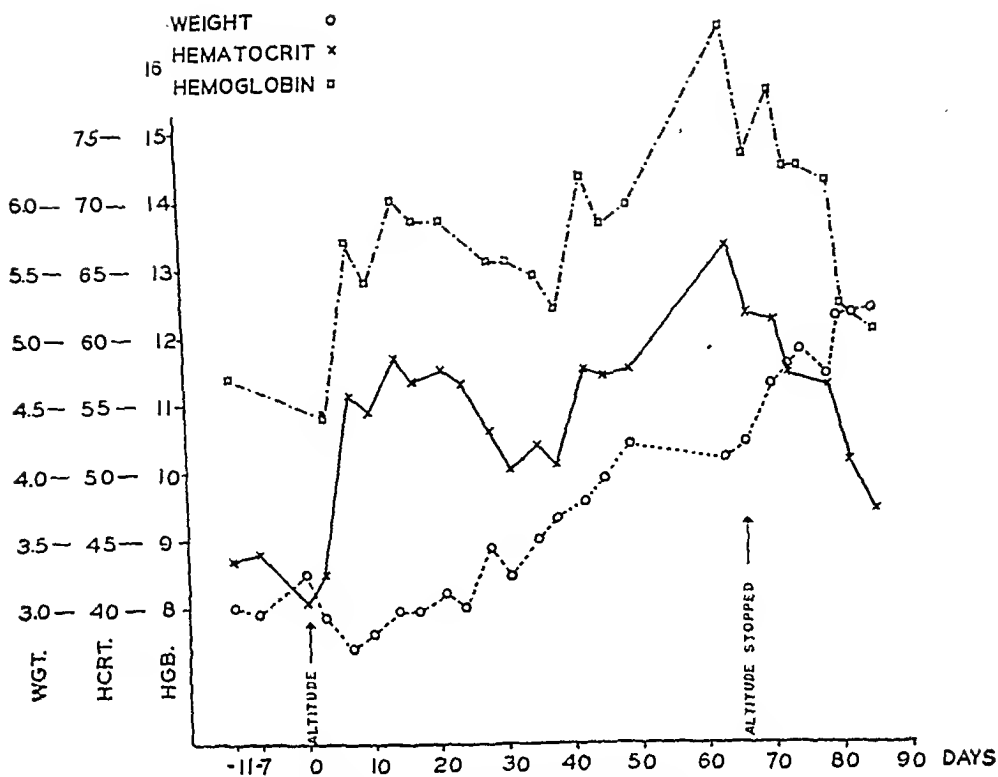


FIGURE 2 EFFECT OF ALTITUDE

Weight, hematocrit, and hemoglobin averages of rabbits having had ten weeks of intermittent altitude exposure.

jections were stopped, was slower than that found with the altitude rabbits.

Two of the 3 animals receiving combined altitude and cobalt treatment died; one apparently from the toxic effects of the cobalt, the other from the combination of toxic effects and peritonitis. Sterile abscesses developed on all 6 animals receiving cobalt injections at or near the site of the injection. The larger of these were opened and drained; however, no infections developed in these areas. The curves for the one surviving altitude and cobalt animal are shown in Fig. 4. They closely resemble those found in the Group II or altitude only animals, with the exception of the absence of the depression of hemoglobin and hematocrit values from 25th to 35th day. This may be due to individual variation. The differences are not sufficient to indicate that the 2 stimuli are

additive in producing polycythemia. However, a more prolonged experiment might show this to be true.

The plasma volume and whole blood volume data are given in Table I, as the average of each group in per cent increase above the prestimulus period. In this manner each animal is compared to himself at the start of the experiment, and as all the animals were still growing all values are positive. The significance can then be shown by comparing the various groups to the control group. The whole blood volume values were obtained from the plasma volume and the corresponding hematocrit value.

The values in parentheses show the per cent change found during each period (*i.e.* the increase or decrease in percentage of each period as compared with that of the preceding period). From this one can more easily

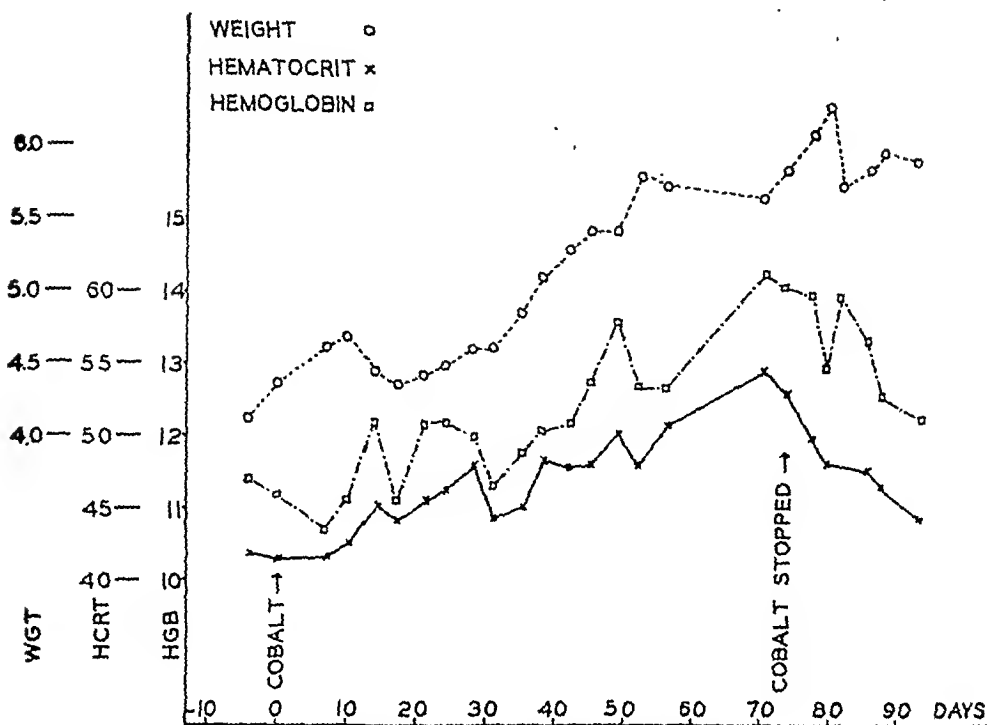


FIGURE 3 EFFECT OF COBALT

Weight, hematocrit, and hemoglobin averages of rabbits given daily cobalt injections.

follow the progressive changes at each period of the experiment.

After 3 weeks of stimuli (Table I, Column 4) there was no significant increase in whole blood volume above that of the controls. However, the hematocrits of those exposed to altitude showed an increased 45% in red cell volume at the expense of the normal increase in plasma volume. The animals exposed to altitude showed almost no change in plasma volume (3% as compared to 50% for the controls, Table I, Column 1).

During the ensuing 7 weeks of exposure to stimuli, the increase of plasma volume in the experimental animals was comparable to the increase in the controls (from 22.5% increase to 32.1% increase). At the same time the increase in the whole blood volume of the altitude animals (66% and 68%) was more than double the increase of the controls (24.2%). This suggests that the development of polycythemia may have 2 phases or processes; an initial immediate and

rapid one involving an increase in the cellular phase at the expense of the plasma volume and a second more gradual step during which the plasma volume increases at a normal rate together with a further increase in the red cell volume.

The changes occurring after removal of the stimuli are for the most part in the red cell component of the blood. The plasma volume of the altitude animals increased above that of the controls and there is a large drop in the total blood volume. The behavior of the altitude plus cobalt animals was similar to that of the controls as far as the plasma volume changes were concerned but there was a large drop in whole blood volume.

Even after 10 weeks of cobalt injections Group III did not have a greater whole blood volume than that of the controls. However, they showed only a moderate polycythemia which was attained at the expense of the plasma volume. Yet after the stimulus had

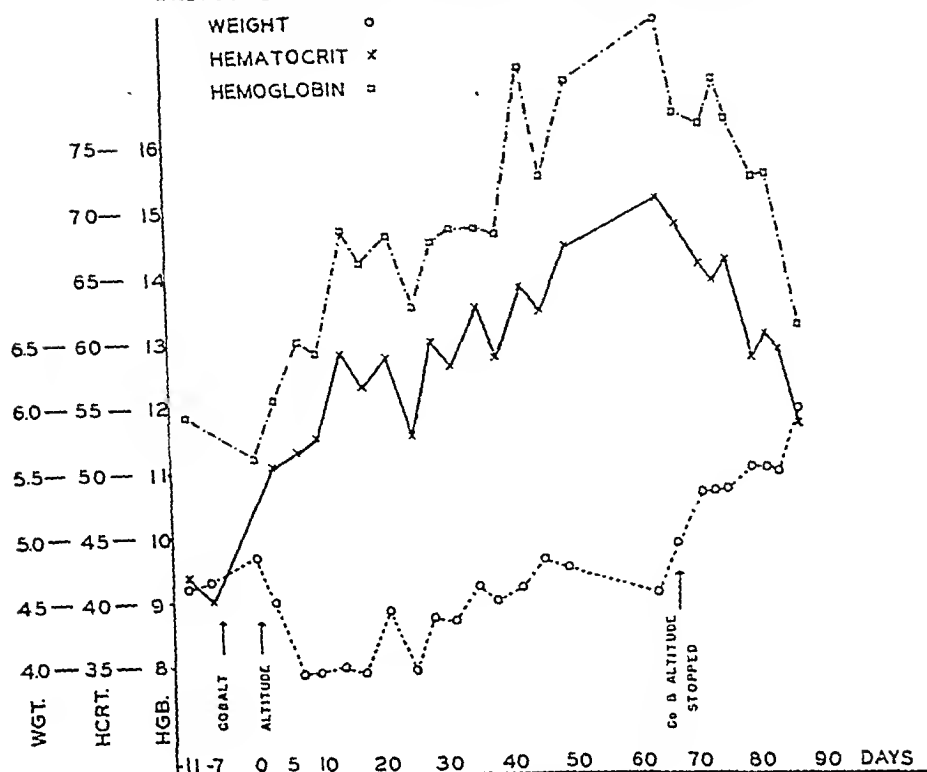


FIGURE 4 EFFECT OF COBALT AND ALTITUDE

Weight, hematoerit, and hemoglobin values of rabbit surviving ten weeks of intermittent altitude and cobalt.

TABLE I.
Percent Increase Above the Pre-experimental Value.

Column No.	Plasma volume			Whole blood volume		
	After 3 weeks of stimulus	10 wks of stimulus	2½ wks of recovery	After 3 wks of stimulus	10 wks of stimulus	2½ wks of recovery
Control	+50.0%	+72.5% (+22.5) [*]	+86.0% (+13.5)	+39.6%	+63.8% (+24.2)	+74.5% (+10.7)
Cobalt and altitude	+3.3	+28.0 (+24.7)	+33.0 (+5.0)	+40.4	+107.0 (+66.6)	+60.0 (-47.0)
Altitude	+2.6	+34.7 (+32.1)	+57.7 (+23.0)	+33.7	+102.2 (+68.5)	+69.3 (-32.9)
Cobalt	+12.3	+40.5 (+28.2)	+33.5 (-7.0)	+26.0	+63.3 (+37.3)	+40.0 (-23.3)

* Values in parentheses show the changes in percent increase above the values of the previous period.

been stopped, there was a decrease in plasma volume as well as total red cell volume.

This either indicates that the development of cobalt and altitude polycythemia involves different mechanisms of response, or that the cobalt animals at 10 weeks had only de-

veloped to a stage comparable to the first 3 weeks of altitude exposure.

Conclusion. Polycythemia can be rapidly induced in rabbits by exposure to altitudes of 20,000 feet 16 hours a day, 5 days a week. It would appear that there is no additive

effect when cobalt injections are combined with the altitude exposure.

There may be 2 stages in the development of polycythemia. The first phase is characterized by a normal whole blood volume with a subnormal plasma volume. The second phase shows a normal rate of increase

in plasma volume with a further increase in whole blood volume.

Subcutaneous cobalt injections slowly induce polycythemia in rabbits but they are accompanied by anorexia, sterile abscesses and other symptoms of toxic effects.

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Effect of Streptomycin on Growth of Rickettsiae in Eggs.

HERBERT R. MORGAN, DAVID A. STEVENS, AND JOHN C. SNYDER.

From the Laboratories of the International Health Division, Rockefeller Foundation and the United States of America Typhus Commission, War Department, Washington, D.C.

In experimental infections, rickettsiae have been shown to be susceptible to the action of penicillin¹ as well as to other therapeutic agents.²⁻⁶ In view of the increasing availability of streptomycin, it was of interest to determine its effect on the growth of representative pathogenic rickettsiae. Using the yolk sac technique of Cox,⁷ the action of streptomycin on the growth of the rickettsiae of the Wilmington strain of murine typhus, the Breinl strain of epidemic typhus and the Karp strain of tsutsugamushi disease in embryonated eggs was studied and compared with the action of *p*-aminobenzoic acid (PABA).

Methods. Streptomycin dissolved in 0.85% saline was sterilized by filtration through a Seitz filter previously washed with distilled

water. A sterile solution of PABA in buffered saline was also prepared. The concentration of these 2 solutions was so adjusted that the dosage employed was contained in 0.4 ml, which was inoculated into the yolk sac of 7-day-old embryonated eggs. The same volume of sterile buffered saline was inoculated into control eggs. Two hours later, both treated and control eggs were inoculated by the same route with a suspension of yolk sacs infected with the Breinl strain of *Rickettsia prowazeki* or the Wilmington strain of *R. mooseri* or the Karp strain of *R. orientalis*. The infective suspensions had been previously titrated in eggs; a dilution was selected which just permitted the survival of a majority of the embryos for 7 days. Uninfected control eggs received injections of the same amounts of streptomycin as a check on its toxicity. The activity of the streptomycin was checked by testing dilutions of the stock solution against a susceptible strain of *Escherichia coli*. Some of the eggs received a second injection of streptomycin 72 hours later.

The eggs were incubated at 35°C for 7 days and candled daily; the dead eggs were discarded. Deaths occurring before the 3rd day were considered to be nonspecific and were disregarded. On the 7th day of incubation, the yolk sacs from all survivors in each group were harvested, divided into

¹ Greiff, D., and Pinkerton, H., *Proc. Soc. Exp. Biol. and Med.*, 1944, **55**, 116.

² Snyder, J. C., Maier, J., and Anderson, C. R., Report to the Division of Medical Sciences, National Research Council, Washington, D.C., Dec. 26, 1942.

³ Greiff, D., Pinkerton, H., and Moragues, V., *J. Exp. Med.*, 1944, **80**, 561.

⁴ Hamilton, H. L., Plotz, H., and Smadel, J. E., *Proc. Soc. Exp. Biol. and Med.*, 1945, **58**, 255.

⁵ Hamilton, H. L., *Proc. Soc. Exp. Biol. and Med.*, 1945, **59**, 220.

⁶ Smadel, J. E., Snyder, J. C., Jackson, E. B., Fox, J. P., and Hamilton, H. L., (Abstract), *Fed. Proc.*, 1946, **5**, 254.

⁷ Cox, H. R., *Pub. Health Rep.*, 1938, **53**, 2241.

STREPTOMYCIN ON GROWTH OF RICKETTSIAE

TABLE I.

Effects of Streptomycin and *p*-Aminobenzoic Acid on the Multiplication of *Ecketsia pronazeki*, *R. mooseri*, and *R. orientalis* in Embryonated Hens' Eggs.

Strain	Experiment	Drug	Dose for each egg, mg	Interval between therapy and infection, hr	Ratio surviving embryos to total in groups at 7th day	No. of viable rickettsiae per ml of yolk sac pools*
<i>R. pronazeki</i> (Breml strain)	I	Streptomycin	0.5	-2	13/15	105.3, 105.3
		PABA	0.5	-2, +72	11/15	107.3, >108.3
		Saline	0.5	-2	11/14	<101.3
		Streptomycin	5.5	-2, +72	7/9	108.3, 108.6
		Saline	5.5	-2	11/13	>105.3
	II	Streptomycin	0.5	-2	7/9	107.8, >108.3
		Saline	0.5	-2	11/12	107.6, >108.3
		Streptomycin	1.0	-2	14/15	108.6
		Saline	2.0	-2	13/15	105.9, 107.3
		Streptomycin	2.0	-2	9/12	108.3, 108.8
<i>R. mooseri</i> (Withington strain)	III	Streptomycin	2.0	-2	9/12	108.2, 108.3
		Saline	2.0	-2	11/13	<101.3
		Streptomycin	11.0	-2	4/8	107.6, 107.3
		Saline	11.0	-2	13/14	105.3, 105.0
		Streptomycin	2.0	-2	9/10	101.7
	IV	PABA	2.0	-2	11/13	105.3, 105.0
		Saline	2.0	-2	4/8	101.7
		Streptomycin	11.0	-2	12/13	105.8, 105.6
		Saline	11.0	-2	12/13	105.3, 105.0
		Streptomycin	2.0	-2	12/13	105.3, 105.0
<i>R. orientalis</i> (Karp strain)	V	PABA	2.0	-2	11/13	105.3, 105.0
		Saline	11.0	-2	12/13	105.3, 105.0

* Numbers estimated from 50% end points in animal tests. See text for description of methods. When 2 figures are given, they represent values from duplicate determinations. The symbols > and < indicate that the end points fell beyond the titration dilutions employed.

2 lots each containing 3 to 7 sacs, and shaken in bottles with glass beads and an appropriate amount of sterile diluent (nutrient broth for *R. prowazeki* and *R. mooseri* and skim milk for *R. orientalis*) to make a 50% suspension and stored in sealed glass ampoules in a carbon dioxide cabinet.

Subsequently these yolk sac suspensions were thawed, lightly centrifuged, and diluted in broth or skim milk for injection into animals. The serial 10-fold dilutions of yolk sacs infected with the Breinl strain of *R. prowazeki* were inoculated intraperitoneally into 4 cotton rats for each dilution. Three weeks later, all of these rats were tested for immunity by the intracardial inoculation of approximately 4 "certainly fatal doses" of yolk sacs infected with the Breinl strain. The 50% immunizing end-point of the treated and control yolk sac pools was calculated⁸ on the basis of the immunity produced in cotton rats in such challenge experiments.

The yolk sac pools infected with the Wilmington strain of *R. mooseri* were titrated by injecting 0.25 ml of the serial 10-fold dilutions in nutrient broth into each of the 8 albino mice, by the intraperitoneal route. Three weeks after injection, the surviving mice were tested for immunity by the intravenous injection of 0.2 ml of an infected yolk sac suspension containing 4 LD₅₀ "toxic doses." The 50% immunizing end-points of the drug-treated and control yolk sac pools were calculated on the basis of the number of mice surviving 10 days after the challenge.

Titration of the yolk sac pools infected with the Karp strain of *R. orientalis* were carried out by injecting 0.25 ml of the serial 10-fold dilutions prepared in skim milk intraperitoneally into albino mice using 8 mice per each dilution. The mice were observed for 21 days, and the 50% end-points were calculated on the basis of the survivors in each group of 8 mice.

For convenience in evaluating results the minimal quantity of viable rickettsiae required to produce immunity in cotton rats (*R. prowazeki* and *R. mooseri*) or death in

white mice (*R. orientalis*) is assumed to be one viable organism. It is appreciated that the minimal quantity may be more than one organism, but for the purpose of comparison in these tests, this point is disregarded.

Results. The data are summarized in Table I. Streptomycin appears to have some inhibiting effect on the growth of *R. prowazeki* in embryonated eggs. The "numbers of viable rickettsiae" in eggs infected with *R. prowazeki* and injected with 0.5 mg of streptomycin were 10^{5.3} and 10^{6.3}, as compared with 10^{8.3} and 10^{9.0} in the control eggs. A second injection of 0.5 mg, 72 hours after infection did not increase this effect. Increasing the amount of streptomycin to 1.0 and 2.0 mg did not appear to increase the inhibitory effect. The mean value of the numbers of *R. prowazeki* in streptomycin-treated eggs in the 3 experiments (7 titrations which properly spanned the end-points) was 10^{6.9} as compared with the mean value of 10^{8.6} attained in control eggs (5 titrations). The difference is statistically significant (Student's "t" test; P lies between 0.05 and 0.02).

In the case of *R. mooseri*, the mean value for the eggs receiving 2.0 mg of streptomycin was 10^{6.3} rickettsiae as compared with 10^{7.5} for the controls. This difference would be expected to occur by chance only once in more than 20 times.* These effects are small when compared to the striking reduction of growth of both *R. prowazeki* and *R. mooseri* with PABA.

Streptomycin in doses of 2.0 mg per egg had no significant effect on the growth of *R. orientalis* in embryonated eggs. This is demonstrated by the "numbers of viable rickettsiae" in yolk sacs from treated eggs, 10^{5.3} and 10^{5.0}, as compared with 10^{3.3} and 10^{5.6} in the untreated controls. These results with 2.0 mg of streptomycin are in contrast with those obtained with 11 mg of PABA which produced a 10,000-fold decrease in the amount of growth obtained with *R. orientalis*.†

* The technique of titration and the reproducible nature of the results obtained with it will be discussed in detail in another publication.

⁸ Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, 27, 493.

Two experiments were performed to determine whether streptomycin was rapidly inactivated in the chick embryo and thus might appear to have little effect on rickettsial growth when compared with PABA (which is known to remain at adequate levels in the egg for the period of time covered by these experiments.)" Streptomycin does not appear to be inactivated, since allantoic fluids and yolks harvested 4 days and 7 days after the inoculation of eggs with 1.0 mg by the yolk sac route were shown to contain the expected amounts, that is, between 5 and 25 μ g per ml, by assay with 3 strains of organisms susceptible to streptomycin, *E. coli* (Waksman) and *Bacillus friedländeri* (Yarsik) and *B. friedländeri* (Lundgren).

Discussion. The concentrations of streptomycin in these tests were selected to represent approximately the concentrations attainable in man. With therapeutic effects in eggs of such a slight degree, it seems unlikely that streptomycin would have any very striking effect on human infections caused by *R. prowazeki*, *R. mooseri*, or *R. orientalis*.

† The minimum concentration of PABA required to produce a detectable inhibition of the growth of *R. orientalis* is considerably greater than that required for inhibition of *R. prowazeki*.⁹

⁹ Snyder, J. C., and Stevens, D. A., unpublished observations.

The effects are sufficient, however, to caution against the use of streptomycin in the attempt to isolate *R. prowazeki* or *R. mooseri* from material contaminated with streptomycin-sensitive bacteria, particularly if the numbers of rickettsiae in the material are small.

Summary. Streptomycin in amounts of from 0.5 to 2.0 mg injected into infected embryonated eggs has a definite slight inhibitory action on the growth of *R. prowazeki*. In the case of *R. mooseri*, 2.0 mg of streptomycin had a small but significant effect. With *R. orientalis*, a dose of 2.0 mg per egg appeared to have no significant effect on the multiplication of the rickettsiae. By contrast, appropriate doses of PABA (5.5 mg per egg for *R. prowazeki*, and 11.0 mg per egg for *R. mooseri* and *R. orientalis*) produced a striking inhibition of growth, reducing the numbers of viable rickettsiae by 10,000 to over 10,000,000 times as compared with untreated controls.

Addendum. As this manuscript was in preparation, Dr. J. E. Smadel informed the authors that his experiments showed a very definite therapeutic effect of streptomycin against infection of embryonated hens' eggs with *R. prowazeki* when doses of 10 mg (10,000 units) per egg were employed, 5 times the maximum dose administered in the experiments herein reported.

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Action of Curare on Temperature Changes in the Brain in Combination With Pentobarbital Narcosis.

SERGEI FEITELBERG AND ERNEST P. PICK.*

From the Laboratories of the Mount Sinai Hospital, New York.

It has been demonstrated that curare-alkaloids (Merck strychnos curare, crystallized tubocurarine hydrochloride) and dihydroerythroidine bromide have a central action, besides the peripheral effect on the neu-

romuscular junction.^{1,2} The central action of these alkaloids was first demonstrated on the electroencephalogram of frogs. At first the amplitude and the frequency of the action

¹ Feitelberg, S., and Pick, E. P., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 654.

² Pick, E. P., and Udda, K., *J. Pharm. and Exp. Therap.*, 1945, **83**, 59.

* Aided by a grant from the Ella Sachs Plotz Foundation for Advancement of Scientific Investigation.

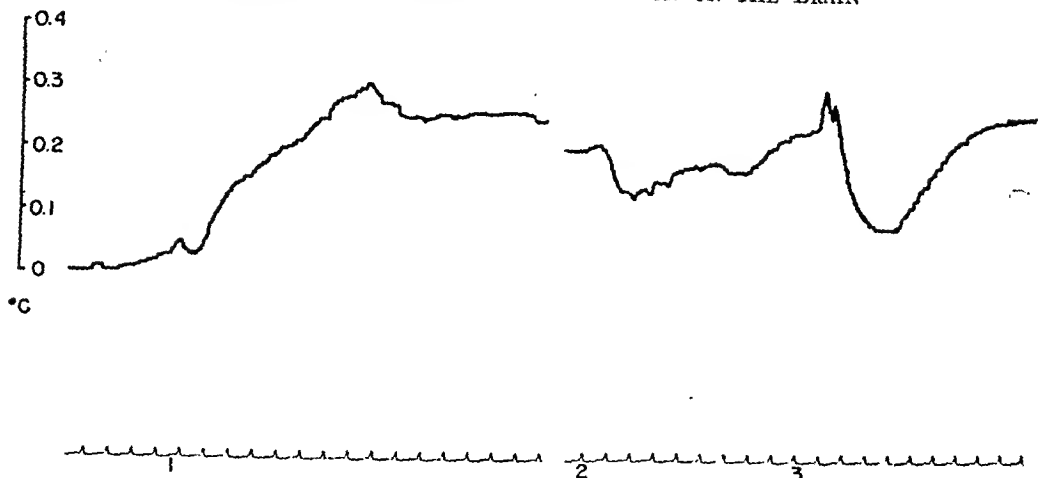


FIG. 1.

Effect of strychnos curare on temperature in the brain during light pentobarbital narcosis. Cat, 3.0 kg, narcotized with pentobarbital 15 mg/kg intraperitoneally and given artificial respiration. Time interval: 1 minute; temperature interval: 0.1°C.

(1) 2 mg strychnos curare intravenously causes a rise of 0.3°C in the brain temperature, which lasts about 8 minutes.

(2) 30 mg per kg pentobarbital intraperitoneally causes a slight drop of the brain temperature which returns to original level in 4 minutes.

(3) 2 mg strychnos curare causes a sudden drop in temperature of the brain (0.2°C) lasting about 4 minutes, which returns to normal after about 6 more minutes.

potentials are diminished. Gradually, they disappear completely. The same behavior of the electrical activity in the brain is observed in narcosis. This central action is independent of the peripheral paralysis. It seems desirable to investigate the effect of curare on the brain of mammals with the method of measurement of temperature changes in the brain, developed by Feitelberg and Lampl.³ Since it has been shown by Pick and Richards⁴ that pentobarbital and curare (and curare-like alkaloids) have a synergistic hypnotic action it is interesting to examine to what extent the temperature changes in the brain is influenced by curare in pentobarbital narcosis of varying depth.

The experiments were carried out on cats (average weight 2.5 to 3.5 kg), in pentobarbital narcosis of varying depth, under artificial respiration. Curare (Merck strychnos curare, 1.5 mg per cc of saline solution) was administered intravenously. We began the experiments with light pentobarbital

narcosis (15-20 mg/kg); in other experiments we used larger doses of pentobarbital (30 mg/kg) to produce a deeper narcosis. The temperature changes were measured by a method described in detail elsewhere.³ This method consists essentially in the following: one junction of a thermocouple is introduced into the internal carotid artery, and the other junction into the brain; the difference of temperature between the 2 junctions (between the brain and the carotid blood) is measured by a galvanometer of adequate sensitivity; this difference of temperature is interpreted as a measure of oxidation processes in the brain. The obvious objection that the difference in temperature is influenced by change in blood flow and not by temperature changes in the brain has been discussed elsewhere.³ It suffices to mention here that the experimental findings on the behavior of temperature changes in the brain cannot be explained only by circulatory processes eventually produced by curare.

³ Feitelberg, S., and Lampl, H., *Arch. f. exp. Path. and Pharm.*, 1935, 177, 600, 725.

⁴ Pick, E. P., and Richards, G. V., in print, *J. Pharm and Exp. Ther.*, 1947.

1. Combined action of curare and light pentobarbital narcosis. Most of our experiments on animals in light pentobarbital an-

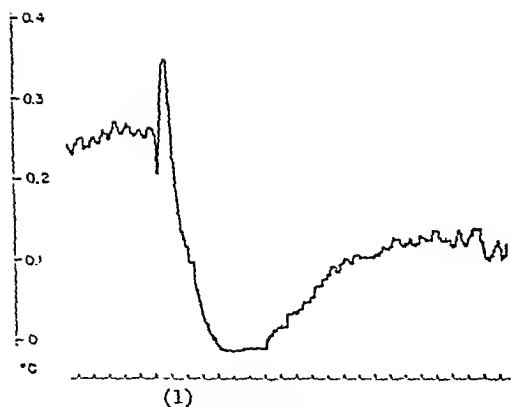


FIG. 2.

Effect of strychnos curare on temperature in the brain during deep pentobarbital narcosis and artificial respiration. Cat, 3.5 kg, narcotized with pentobarbital 30 mg/kg intraperitoneally. Time interval: 1 minute; temperature interval: 0.1°C. At (1) 3 mg strychnos curare intravenously causes a sudden drop in the brain temperature corresponding to 0.25°C, lasting about 7 minutes.

esthesia show that administration of curare causes a rise in temperature of the brain (Fig. 1). This indicates that curare, given in light narcosis without complete peripheral paralysis, has a stimulating effect on the brain. The increased temperature lasts for 10 to 15 minutes. This effect can be elicited several times on the same narcotized animal. It is independent of the peripheral body temperature. It may well be the result of actual increase in brain metabolism.

2. *Combined action of curare and deep pentobarbital narcosis.* The effect of curare is reversed if the pentobarbital narcosis is deeper, when the peripheral muscle movements are abolished by the narcosis. Under those conditions curare causes a marked decrease in temperature of the brain, lasting 5 to 15 minutes, which may be interpreted as a depression of the oxidation processes in the brain (Fig. 2). It appears therefore that a certain depth of pentobarbital narcosis is necessary for the occurrence of synergism between curare and pentobarbital narcosis. No synergism seems to exist when the pentobarbital narcosis is light.

3. *Discussion.* The stimulating and depressing action of curare which we found in experiments on cerebral temperature is in

agreement with the experiments reported by McIntyre *et al.*,⁵ on the electroencephalogram of dogs in light pentobarbital narcosis after intravenous injection of *d*-tubocurarine. The initial effect of curare was an increase of electrical brain activity in the occipital, parietal and frontal region with a 3-fold increase in amplitude and occasional bursts of high frequency. This stage was followed shortly by a depression of electrical activity. With larger doses the depression occurred earlier and the stage of initial stimulation was brief. The latter findings agree with the earlier statements of Feitelberg and Pick¹ and Pick and Unna² about the depressing action of curare and curare-like alkaloids on the electroencephalogram of frogs.

The central stimulating action of curare producing hyperexcitability, accelerated respiration, central vagus stimulation and clonic convulsions was also found recently in experiments with potcurare and purified curarine chloride in cats by v. Euler and Wahlund⁶ and in experiments with nonfatal doses of intocostarin and crystalline *d*-tubocurarine chloride on various mammalian species by Cohnberg;⁷ central nervous depressants, *i.e.*, sodium amytal or cyclopropane decrease, abolish or prevent the stimulating central effect.

It is further interesting to note that in earlier investigations of the action of *d*-tubocurarine on frogs, Tillie⁸ and Jacobhazy⁹ have described a stimulation of the brain followed by a general reflex inhibition and paralysis of the spinal cord. According to Fuehner¹⁰ guanidine, which has curare-like properties, produces also an initial central

⁵ McIntyre, A. R., Dunn, A. L., and Tullar, P. E., *Fed. Proc.*, Part II, **67**, February, 1946.

⁶ v. Euler, U. S., and Wahlund, H., *Acta physiol. Scandinav.*, 1941, **2**, 327.

⁷ Cohnberg, R. E., *J. Lab. and Clin. Med.*, 1946, **31**, 566.

⁸ Tillie, J., *Arch. f. exp. Path. und Pharm.*, 1890, **27**, 1.

⁹ Jacobhazy, S., *Arch. f. exp. Path. und Pharm.*, 1899, **42**, 10.

¹⁰ Fuehner, H., *Arch. f. exp. Path. und Pharm.*, 1908, **58**, 1.

excitation in frogs, followed later by a central paralysis, which occurs 2 hours before peripheral paralysis appears.

4. *Summary:* Curare, which affects the electrical activity of the brain has also a distinct effect on the temperature in the brain in combination with pentobarbital narcosis.

These temperature changes in the brain indicating changes of oxidation processes are increased by curare under light pentobarbital narcosis, and decreased under deep pentobarbital narcosis. This latter central action may be involved in the synergistic narcotic effect.

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Growth of Chicks on Purified and Synthetic Diets Containing Amino Acids.*

T. D. LUCKEY, P. R. MOORE, C. A. ELVEHJEM, AND E. B. HART.

From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison.

Although amino acids have been used in recent investigations¹⁻³ as the principal nitrogen source in chick diets in order to determine the amino acid requirements of the chick, such diets have various other applications in nutritional investigations. We have fed amino acid diets in an attempt to determine (1) if the amino acids present in casein can be replaced by an equivalent amount of free amino acids; (2) if chicks will grow as well on a mixture of essential amino acids as when additional nonessential amino acids are added; (3) if chicks require streptogenin;^{4,5} (4) if chicks will survive and develop normally on a purely synthetic diet (a chemically-defined diet consisting of C.P. chemicals); and (5) if a chemically-defined diet can be used effectively in the elucidation of unknown factors such as the monkey antianemia factor of Cooperman *et al.*⁶

Experimental. Day-old White Leghorn chicks weighing 40-42 g were divided into groups of 3 chicks each and maintained in electrically-heated cages with raised screen bottoms. In Series 1 and 2 (Table II) the chicks were fed a protein and amino acid-free diet for 3 to 4 days (to deplete them of any possible nitrogen stores carried in the yolk sac) prior to feeding the experimental diets. Since this preliminary depletion period appeared to inhibit later growth responses it was discontinued and the animals were placed directly on the experimental diets in subsequent experiments.

The composition of the diets used is given in Table I. Diet I is a typical experimental diet with 18% casein supplemented with arginine, glycine, and cystine and adequate amounts of the vitamins known to be involved in chick nutrition. Diet II is similar to Diet I but contains free amino acids equivalent to the amount present in casein with arginine, glycine, and cystine additions as in Diet I. Diet III contains the amino acids essential to the chick in amounts adequate to equal the nitrogen contents of Diets I and II. Diets IV, V and VI represent

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from the Wisconsin Alumni Research Foundation and Swift and Company.

¹ Almquist, H. J., and Grau, C. R., *J. Nutr.*, 1944, **28**, 325.

² Hegsted, D. M., *J. Biol. Chem.*, 1944, **156**, 247.

³ Grau, C. R., and Peterson, D. W., *J. Nutr.*, 1946, **32**, 181.

⁴ Woolley, D. W., *J. Biol. Chem.*, 1945, **159**, 753.

⁵ Patton, A. R., Marvel, J. P., Petering, H. G., and Waddell, J., *J. Nutr.*, 1946, **31**, 485.

⁶ Cooperman, J. M., Waisman, H. A., McCall, K. B., and Elvehjem, C. A., *J. Nutr.*, 1945, **30**, 45.

⁷ Briggs, G. M., Jr., Luckey, T. D., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1944, **153**, 423.

TABLE I.
Diet Composition in Percent.

Constituent	I	II	III	IV	V	VI	VII
Dextrin*	61.0	61.0	61.0	33.0	33.0	33.0	
Cerelose				33.0	33.0	33.0	
Celluloflour†				3.0	3.0	3.0	
Glucose C.P.							71.0
Soybean oil	5.0	5.0	5.0	5.0	5.0	5.0	
Salts V‡	6.0	6.0	6.0	6.0	6.0	6.0	6.0
Haliver oil§	.04	.04	.04	.04	.04	.04	
B-vitamins¶							
Calcium gluconate				0.4	0.4	0.4	0.5
Casein (SMA)	18.0				8.0	14.0	
<i>d,l</i> -Alanine		1.23		0.2	0.1		0.2
<i>l</i> (+)-Arginine HCl	0.6	1.59	2.4	1.2	0.6	0.6	1.8
<i>d,l</i> -Aspartic acid		1.38		0.2	0.1		0.2
<i>l</i> (+)-Cysteine HCl		0.09					
<i>l</i> (-)-Cystine	0.3	0.39		0.4	0.2	0.4	0.3
<i>l</i> (+)-Glutamic acid		5.00		1.5	0.75		1.5
Glycine	2.0	2.11	2.0	1.0	0.5	1.0	1.8
<i>d,l</i> -Histidine HCl · H ₂ O		0.68	1.2	0.6	0.3		0.2
<i>d,l</i> -Homocystine		0.05					
<i>l</i> (-)-Hydroxyproline		0.05		0.1	0.05		
<i>d,l</i> -Isoleucine		2.85	4.0	2.0	1.0		2.0
<i>l</i> (-)-Leucine		2.66	3.0	1.5	0.75		1.5
<i>l</i> (+)-Lysine HCl		1.88	2.5	1.2	0.6		1.8
<i>d,l</i> -Methionine		0.77	2.0	1.0	0.5		1.5
<i>d,l</i> -Norleucine		0.05					
<i>d,l</i> -Phenylalanine		1.13	3.0	1.0	0.5		2.0
<i>d,l</i> -Proline		1.80		0.2	0.1		0.2
<i>d,l</i> -Serine		0.11		0.2	0.1		0.15
<i>d,l</i> -Threonine		1.72	4.0	1.4	0.7		2.0
<i>l</i> (-)-Tryptophane		0.26	2.0	0.5	0.25		0.5
<i>l</i> (-)-Tyrosine		1.40		0.5	0.25		1.0
<i>d,l</i> -Valine		2.94	4.0	2.0	1.0		2.0
Total Nitrogen**	3.38	3.38	3.36	1.87	2.20	2.59	2.36

* Corn starch moistened with water and autoclaved at 15 lb for 2 hr, dried, and ground.

† A commercial cellulose product.

‡ Diets IV, V, and VI contained 0.5% Na₂PO₄ and diet VII contained NaHCO₃ 1.0%, Na₂HPO₄ 0.5%, Al₂(SO₄)₃ 0.1%, Na₂B₄O₇ · 10H₂O 0.1%, V₂Cl₂ 0.05%, NiSO₄ 0.03%, Co(NO₃)₂ 0.03%, and K₂SiO₄ 0.1% in addition to salts V.§ Haliver oil fortified with vitamin D₃ to supply .006 mg/100 g ration.

¶ Diets I, II, and III contained α-tocopherol 0.6 mg, 2-me-1,4-naphthoquinone 0.1, thiamine Cl 0.5, riboflavin 1.2, nicotinic acid 10.0, calcium pantothenate 4.0, pyridoxine HCl 0.8, choline Cl 200.0, inositol 100.0, biotin 0.04, and folic acid 0.5 mg per 100 g of ration.

Diets IV, V, and VI contained double the above amounts of α-tocopherol, thiamine Cl, nicotinic acid, riboflavin, calcium pantothenate, pyridoxine HCl and biotin and 2-me-1,4-naphthoquinone 0.1, ascorbic acid 100 mg, *p*-aminobenzoic acid 5.0, folic acid 0.05, and *i*-inositol 100 mg per 100 g of ration and 0.2 mg choline per 100 g of ration. Diet IV contained 200 and diets V and VI 300 mg respectively of choline Cl per 100 g of ration.Diet VII contained carotene 0.6, ascorbic acid 100, vitamin D₃ .005, α-tocopherol 33, 2-me-1,4-naphthoquinone 2.0, thiamine Cl 0.5, riboflavin 1.0, nicotinamide 10.0, Ca pantothenate 2.0, pyridoxine Cl 1.0, pyridoxal Cl 0.1, β-pyrazin Cl 0.5, choline Cl, 200.0, *i*-inositol 100.0, biotin 0.05, folic acid 0.05, and *p*-aminobenzoic acid 10.0 mg per 100 g of ration. In addition Diet VII contained ethyl linolate 0.1, tripalmitin 1.0, ribose 0.03, and arabinose 0.5 g and choline acid .2 mg per 100 g of ration.

** Non-utilized forms of amino acids are omitted in this sum.

modifications of amino acid mixtures with and without protein supplementation. Diet VII is a chemically-defined diet that contains additional growth factors and trace elements. All differences in total weights of the nitrogen sources were compensated for by modifying

the level of carbohydrate.

Results. A direct comparison of individual groups within a given series is difficult, but a comparison of similar lots in the first 3 series demonstrates that casein diets (Groups 1, 7 and 12, Table II) produce better growth

TABLE II.
Amino Acid Diets in Chick Growth.

Series No.	Group No.	Diet No. and description	% nitrogen	Days on experiment	G gain per day
1	1	I—casein, arginine, glycine, and cystine	3.38	18	6.7 (3)*
1	2	II—22 amino acids	3.38	18	2.9 (3)
1	3	III—11 essential amino acids	3.36	14	1.0 (2)
2	4	IV—19 amino acids	1.87	10	1.5 (2)
2	5	V—19 amino acids + 8% casein	2.20	10	2.0 (3)
2	6	III—11 essential amino acids	3.36	10	0.6 (3)
2	7	VI—casein, arginine, glycine, and cystine	2.59	10	4.7 (3)
2	8	VI—casein,† arginine, cystine, and glycine	1.30	10	1.8 (3)
3	9	IV—19 amino acids	1.87	8	3.9 (2)
3	10	V—19 amino acids + 8% casein	2.20	8	3.0 (2)
3	11	III—11 essential amino acids	3.36	8	1.6 (2)
3	12	VI—casein, arginine, glycine, and cystine	2.59	8	4.1 (2)
3	13	VI—casein, arginine, glycine, and cystine	1.30	8	2.6 (2)
4	14	VII—chemical diet—no protein	0.00	16	-0.5 (3)
4	15	VII—18 amino acids	2.36	16	0.7 (3)
4	16	VII—16% amino acids + 4% liver powder‡	1.82§	16	1.5 (2)
4	17	VII—16% amino acids + 4% casein	2.45	16	3.0 (3)
4	18	VI—casein, arginine, glycine, and cystine	2.59	16	5.8 (3)
4	19	VI—casein, arginine, glycine, and cystine	1.30	16	1.0 (2)
4	20	VII—12% amino acids + 4% liver powder + 8% casein	2.67	16	2.5 (3)

* Figures in parentheses refer to number of survivors at the conclusion of the experiment.

† Casein, arginine, glycine, and cystine mixture reduced by one-half and replaced by dextrin.

‡ Whole liver powder.

§ Nitrogen content of the whole liver powder is not included in this sum.

responses in chicks than diets containing the 11 essential amino acids plus nonessential amino acids (Groups 2, 4 and 9) and that the latter diets are in turn superior to diets containing the 11 essential amino acids alone (Groups 3, 6 and 11). Growth differences between similar groups in the different series are not significant since different depletion technics and different experimental times were involved. Furthermore, although uniform chicks were used in any given series, dietary differences in the diets of the hens used as the source of eggs could affect the stores of the chicks which would alter their growth responses on similar diets in series run at different times.

Substitution of one-half of the weight of the amino acids in Diet IV with an equivalent weight of casein (8%) (Groups 5 and 10) produced no better growth responses than the amino acid diets (Groups 4 and 9) or a casein diet in which the nitrogen level was reduced 50% by feeding one-half of the casein and supplementary amino acids of Diet VI (Groups 8 and 13).

Chicks fed an amino acid-free synthetic

diet (Group 14) lived for a remarkably long period and displayed no macroscopic deficiency symptoms other than loss of weight and lethargy. Chicks fed the complete synthetic Diet VII (Group 15) showed poor growth responses. Addition of 4% whole liver powder at the expense of the amino acids doubled the growth response (Group 16). The use of casein in place of whole liver powder gave even better growth responses in the chicks (Group 17) while the addition of both liver powder and casein (Group 20) appeared to produce no better growth response than 4% casein alone.

Discussion. The maximum growth responses on our amino acid diets (3.9 and 3 g per day) compare favorably with those of Hegsted (2.7 and 3.5 g per day).² We recognize that an absolute comparison cannot be made since different sources of animals and different experimental periods were involved.

The general failure of the amino acid diets to produce growth responses equal to diets containing casein supplemented with arginine, glycine and cystine can be explained only

partially by the lack of a growth factor since whole liver substance apparently failed to supplement casein (source of strepogenin) on our synthetic diet. Our results do not, however, eliminate an extremely labile growth factor absent from the whole liver powder used. It is doubtful that the growth differences are due to missing trace elements since the whole liver powder would probably carry adequate amounts of any unidentified essential minerals.

Growth inhibition due to toxicity of some of the *d*-isomers is debatable. Replacement of part of the amino acids in the diet containing the 19 amino acids by casein failed to produce a significant growth response in chicks over a reduced casein diet (Groups 5, 8, 10 and 13) which would be expected if toxic *d*-isomers were involved. This occurred in spite of the fact that some of the amino acids were borderline in the reduced casein diet. Alternatively, the poor growth responses obtained on the diet containing the 11 amino acids (3.36% nitrogen) when compared to the diets containing the 19 and 22 amino acids (2.20 and 3.38% nitrogen respectively) could be explained by the higher percentage of the *d*-isomers in the diet containing the 11 amino acids, or by the possibility that some of the so-called nonessential amino acids are essential on such highly purified diets. It is possible that the problem is physiological rather than nutritional and involves amino acid imbalances other than toxicity of the *d*-isomers, disturbed osmotic pressure relationships, interference with buffer mechanisms in the intestine, poor retention in the tract, or impaired absorption. Inhibition of enzymes or beneficial bacteria in the intestinal tract are other possible effects.

The role of strepogenin in the nutrition of

the chick remains obscure. The addition of casein or whole liver powder to our synthetic diet (Diet VII) produced a positive strepogenin-like response on chick growth (Groups 14, 16, 17, and 20) in contrast to the results with the purified diet (Diet IV) where replacement of one-half of the amino acids with casein failed to give increased growth in the chicks. The slightly higher nitrogen content of the supplemented synthetic diets could have been a factor involved. It is entirely possible that factors other than strepogenin were lacking and it is questionable if uniform growth responses to strepogenin would occur on such a deficient diet. There is also the possibility that the chick carries adequate stores of strepogenin and a true strepogenin response could not be expected on such short term experiments necessitated by the high cost of amino acids.

Summary. Chicks fed a completely synthetic (chemically-defined) diet in which amino acids are the sole source of nitrogen show a definite subnormal growth response.

Casein is superior to its component amino acids as a source of nitrogen for the chick.

Diets containing some of the nonessential amino acids are superior to diets containing the 11 amino acids now recognized as essential for the chick.

We gratefully acknowledge our indebtedness to Merek and Company, Inc., Rahway, N.J., for crystalline vitamins; Wilson Laboratories, Chicago, Ill., for gelatin and whole liver powder; Allied Mills, Peoria, Ill., for soybean oil; Lederle Laboratories, Inc., Pearl River, N.Y., for folic acid; Hoffmann-LaRoche, Nutley, N.J., for *l*-proline; E. I. duPont de Nemours and Company, Inc., New Brunswick, N.J., for crystalline vitamin D₃; and Dr. H. R. Snyder of the Department of Chemistry, University of Illinois, Urbana, Ill., for *dl*-serine.

Absence of Lipotropic Activity of Methionine When Pure Amino Acids Were Substituted for Dietary Protein.

CATHARINE S. ROSE, THOMAS E. MACHELLA, AND PAUL GYÖRGY.*

From the Nutritional Service of the Department of Pediatrics and the Gastro-Intestinal Section of the Medical Clinic, School of Medicine, University of Pennsylvania, Philadelphia.

That methionine shows lipotropic activity in preventing the development of fatty livers has been repeatedly demonstrated, but the variation in experimental procedures and in the results obtained has left the magnitude of this effect and the interrelationships of methionine with other lipotropic and anti-lipotropic agents in some question. Lucas and Best¹ have reviewed this subject and have noted that there is disagreement among various workers as to whether the lipotropic action of casein is due solely to its methionine content. It was thought pertinent in this connection to observe the effect of methionine when a mixture of essential amino acids (supplemented with glutamic acid) was substituted for natural protein in the experimental diet.

Experimental. The composition of the amino acid mixture used is shown in Table I. It was designed to contain the essential amino acids (with the exception of methionine) in the proportions in which they occur in casein, glutamic acid making up the nonessential fraction. When the *L*-form was not obtainable, twice the amount of the *DL*-form was used.

TABLE I.
Amino Acid Mixture
(% Composition)

<i>L</i> -Arginine HCl	4.4
<i>DL</i> -Phenylalanine	10.3
<i>DL</i> -Leucine	27.0
<i>DL</i> -Isoleucine	9.7
<i>L</i> -Histidine HCl	2.3
<i>L</i> -Lysine HCl	7.0
<i>DL</i> -Threonine	8.6
<i>DL</i> -Tryptophane	2.4
<i>DL</i> -Valine	10.0
<i>L</i> -Glutamic Acid HCl	18.3

*The authors are grateful to Dr. Harry S. Goldblatt for examining the kidney sections and to Mrs. A. B. Stavitsky for technical assistance.

¹ Lucas, C. C., and Best, C. H., *Vitamins and Hormones*, Vol. I, p. 1, 1943, New York.

Thirty young adult male rats of a Wistar strain were divided into 2 groups of 15 rats each. All animals were fed on a diet consisting of amino acid mixture, 8.5; cane sugar, 47.5; Crisco, 40; salt mixture No. 2 (U.S.P. XII), 4. Each animal received daily by stomach tube 20 μ g thiamine chloride, 100 μ g calcium pantothenate, 20 μ g pyridoxine and 25 μ g riboflavin, dissolved in 1 cc of water. Three drops of percomorph oil furnishing 3750 units of vitamin A and 540 units of vitamin D, and 3 mg of α -tocopherol were given weekly. The methionine-treated group received an additional supplement of 50 mg *DL*-methionine dissolved as the sodium salt in 1 cc of water and administered by stomach tube.²

The rats were maintained on experiment for 21 days. Food intake and body weight were recorded daily. At the end of the experimental period the animals were exsanguinated by decapitation under light ether anesthesia and the livers removed, weighed and analyzed for total fat by the method of Tucker and Eckstein,³ modified slightly. The kidneys were saved for histological examination.

Results and Discussion. The basal diet corresponded in essential amino acid content to one containing about 7% casein and, with the mixture of *L*- and *DL*-acids, to a little less than this in available nitrogen, with no sulphur-containing amino acid present. When supplementary methionine was given, the 50 mg per day represented a level in the diet of 0.8% on the basis of the average food intake of the group, or the same amount of methionine as a diet containing 25% casein.

That the rats should not thrive on the

² Machella, T. E., and Griffith, J. Q., Jr., in Griffith-Farris, *The Rat*, 1942, Philadelphia.

³ Tucker, H. F., and Eckstein, H. C., *J. Biol. Chem.*, 1937, **121**, 479.

sulphur-free diet was to be expected. Weight loss was continuous throughout the experiment averaging 30% for the group. The animals did not, however, exhibit any acute symptoms during the 21-day period. Methionine exerted a beneficial effect on the general condition of the animals. The methionine-supplemented group ate 15% more than the control animals. Weight loss paralleled that of the controls for the first week, but during the remaining 2 weeks of the experiment body weight was almost constant. The average weight loss for the period was 21%. This figure does not compare favorably with the average 12% loss of rats on an unsupplemented 8% casein diet which was observed in this laboratory.[†] This mild effect of methionine on body weight and food intake is in contrast with the results of Glynn, Himsworth and Neuberger.[‡] These authors using a high carbohydrate, very low fat diet, with amino acids as the source of nitrogen, found that with the addition of 8 mg of methionine a day to the sulphur-free ration the rats ate two-thirds more, while 80 mg a day more than doubled the food intake. The variation was in the amount of carbohydrate consumed, the amount of amino acids being kept constant at 1.3 g per day. The animals used weighed 80 g at the beginning of the experiment. With no methionine, weight change in 21 days was -2.5%, with 8 mg of methionine, +2.5%, and with 80 mg of methionine, +44%. These workers were studying pathological changes in the liver. That this might be uncomplicated by fatty infiltration, they included in the diet sufficient choline to prevent fatty livers. Our diet was essentially choline-free which may have necessitated a quite different apportionment of the methionine among its various functions.

No necrosis was observed in any of the livers at autopsy, nor did the kidneys show any pathological changes.

Methionine proved quite ineffective as a lipotropic agent in our experiment. The level

of liver fat in the control group, 19.7% was of the order usually obtained with a similar diet containing casein. The average for the treated animals was slightly lower, 17.9%, but the difference is insignificant. It has been reported many times that a similar amount of methionine supplementing a ration containing 5% casein has definite lipotropic effect. As examples may be cited the early paper of Tucker and Eckstein³ in which it was stated that 0.5% of methionine added to the diet reduced the liver fat from 20.4% to 11.1% and the recent work of Clark, Eilert and Dragstedt⁵ who found the liver fat changed from 31.5% to 9.3% by the same amount of methionine. In both cases the total level of methionine in the diet was lower than in our experiment: about 0.65% as compared with 0.8%. However, because of the better appetite of the animals on the casein diet, the actual amount of methionine ingested was a little greater than that given to our animals. The rats of Tucker and Eckstein received 71 mg per day and those of Clark, Eilert and Dragstedt, 58 mg, while ours had 50 mg.

The results of the present study appear to support the view that other factors than methionine enter into the lipotropic effect of a high casein diet, or even of a methionine-supplemented low casein diet. Under the conditions of the experiment methionine, in what has been considered as an adequate amount, gave no appreciable protection against development of fatty livers. The amino acid mixture used did not reproduce the composition of casein except for the essential amino acids. Some of the nonessential acids may play a role or, in their absence, the requirement for some of the essential acids may be increased. It has been suggested by du Vigneaud, Chandler, Moyer and Keppel⁶ that in low protein diets a deficiency of some other precursor of choline than methionine may be the limiting factor of lipotropic activity. On the other hand,

³ Clark, D. E., Eilert, M. L., and Dragstedt, L. R., *Am. J. Physiol.*, 1945, **144**, 620.

⁶ du Vigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M., *J. Biol. Chem.*, 1939, **131**, 57.

[†] Unpublished data.

[‡] Glynn, L. E., Himsworth, H. P., and Neuberger, A., *Brit. J. Exp. Path.*, 1945, **26**, 326.

TABLE II.
Effect on Body Weight, Food Intake, and Liver Fat of Adding Methionine to a Diet Free of Sulfur-Containing Amino Acids.

Group	Treatment	No. of rats	Wt of animals		Food intake, g/day	Liver	
			Initial, g	Change, %		Wt, g	Total lipid, %
I	Basal diet	15	160 (149-172)	-29.8 ± 0.8	5.5 ± 0.2	5.30 ± 0.24	19.7 ± 1.7
II	Basal diet + Methionine	15	164 (146-182)	-21.0 ± 0.8	6.3 ± 0.2	5.50 ± 0.14	17.9 ± 1.4

the amount of methionine required to show a lipotropic effect may be considerably increased in a diet containing minimal amounts of choline. In the present investigation crystalline vitamin B components were used, while in those quoted above^{3,5} the animals were fed 0.5 g of yeast daily. This amount of yeast contains more than 1.0 mg of choline⁷

⁷ Fletcher, J. P., Best, C. H., and Solandt, O. M., *Biochem. J.*, 1935, 29, 2278.

which is an appreciable percentage of the daily requirement. It is possible, also, that some other component of yeast works in conjunction with methionine.

Summary. Under the experimental conditions chosen, methionine at a level of 50 mg per day (0.8% of the diet) did not prevent the development of fatty livers in rats fed a diet in which a mixture of essential amino acids replaced protein.

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Implantation of Riboflavin Pellets in Animals and Man.

Y. M. BROMBERG, A. BRZEZINSKI, AND F. SULMAN. (Introduced by B. Zondek.)

From the Gynecologic-Obstetric Department, Rothschild Hadassah University Hospital and the Hormone Research Laboratory, Hebrew University, Jerusalem, Palestine.

Nutritional studies of the Palestinian population have shown that riboflavin deficiency is very often observed in this country,¹ and that its incidence is far more frequent than that of other vitamin deficiencies.² The clinical manifestations of this deficiency, such as glossitis, cheilosis, corneal vascularization, etc., were particularly common in pregnant women. Low riboflavin excretion in the urine (averaging 95 γ per liter instead of 360 γ per liter in normal pregnant women) was found in 190 (21%) out of 900 pregnant women studied.³ Furthermore, observations

have been reported as to the harmful effect of riboflavin deficiency on the course of pregnancy (prematurity), on the condition of the fetus *in utero* (higher incidence of antenatal death) and upon the postpartum period (agalactia and hypogalactia).⁴

Surprisingly enough, these clinical manifestations appeared not only in the poorer classes, in which riboflavin intake was inadequate, but also in well nourished subjects. The fairly frequent incidence of riboflavin deficiency in well nourished individuals is principally due to inadequate resorption of this vitamin. The frequent occurrence of various intes-

¹ Dostrowsky, A., and Sagher, Fr. Harefuath, *J. Pol. Med. Assn.*, 1942, 22, 1942; *Dermatologica*, 1942, 86, 325.

² Berger-Rabinowitz, S., Doctor's Dissertation, 1945, Hebrew University, Jerusalem.

³ Braun, K., Bromberg, Y. M., and Brzezinski, A., *J. Obst. and Gyn., Brit. Empire*, 1945, 52, 1.

⁴ Brzezinski, A., Bromberg, Y. M., and Braun, K., *J. Obst. and Brit. Emp.*, 1947, in press.

tinal and hepatic diseases (amebic dysentery, gastric achlorhydria and hypochlorhydria) and various forms of chronic hepatitis is undoubtedly the most important etiologic factor in the production of deficiency symptoms in these subjects. Riboflavin therapy generally gives striking results in those cases in which the deficiency is induced by insufficient intake.^{1,2} On the other hand, poor results are generally observed when riboflavin is administered to subjects suffering from riboflavin deficiency due to inadequate resorption. In such patients the oral administration of riboflavin seems to be ineffective. Furthermore, riboflavin administered parenterally or intravenously, is eliminated from the body very rapidly. Thus, in order to maintain a constant level in the tissues and to prevent the rapid elimination of riboflavin,^{3,4} the daily dose must be divided into many subdoses.

The poor therapeutic results obtained with riboflavin in patients suffering from diseases of the digestive tract led us to attempt the implantation of riboflavin pellets so as to afford adequate and continuous resorption of this vitamin. This method of supply seems to us particularly advantageous in patients who require repeated daily injections of riboflavin for a relatively long period. In addition, the difficulties involved in handling this substance which is easily inactivated when exposed to light, and the unreliability and lack of cooperation of patients, induced us to search means of supplying riboflavin in controlled quantities.

Technic. Pellets implanted in rats. Pellets containing riboflavin* adsorbed on Fuller's earth were used at first but later discarded, because they were completely absorbed in the body within a fortnight. Much better results were obtained when a mixture of 20 mg cholesterol plus 20 mg riboflavin was used. Pellets prepared in this way lost 90% of their riboflavin content within 1-1½ months. In the present report the results obtained with this type of pellet will be described. It seems that better results can be obtained with pellets prepared by melting

together cholesterol with riboflavin. Results with this method will be described elsewhere. Sterilization of the pellets was carried out by heating them twice for 4 hours at 80°C.

Pellets implanted in patients (humans). The pellets implanted in patients were made by mixing 50 mg cholesterol with 50 mg riboflavin. This mixture was made up into 4 pellets. They were sterilized by heating them 3 times for 4 hours at 80°C. The riboflavin was handled in darkness; implantation was carried out subcutaneously in the lateral aspect of the thigh. Four pellets containing 50 mg riboflavin in all were implanted into each patient.

Riboflavin determination in the urine and the recovered pellets was carried out by the microbiological method of Snell and Strong.^{5,†}

Results. Resorption of riboflavin pellets in rats. A sterile pellet containing 20 mg of riboflavin with 20 mg of cholesterol was implanted in the medial muscles of the thigh of 5 female albino rats weighing 150 g each. The rats were kept on a stock diet consisting of sprouted wheat, oats and bran supplemented by milk and vegetables in season. Riboflavin excretion in the urine was determined at intervals of 2 days for 2 weeks preceding the implantation. After the implantation riboflavin excretion was determined every other day, and when the riboflavin level had returned to normal, at weekly intervals. When the rats were sacrificed, the "ghost" of the pellet was recovered and its riboflavin content determined. It contained on an average 20% of the original riboflavin content one month after implantation. The results are compiled in Table I. Table I demonstrates that increased riboflavin excretion persisted for one month after implantation. Later on, riboflavin excretion returned to the normal level, thus proving the discontinuance of any further resorption of riboflavin from the pellet.

Resorption of riboflavin pellets in human patients. At first one normal well nourished

⁵ Snell, E. E., and Strong, F. M., *Ind. and Eng. Chem., Anal. Ed.*, 1939, 11, 346.

[†] We are indebted to Dr. Lea Bichowsky for these analyses.

* We are indebted to "Assia" Chem. Lab., Tel-Aviv, for a generous supply of riboflavin.

TABLE I.
Riboflavin Excretion in the Urine of 5 Adult Female Albino Rats Before and After Intramuscular Implantation of Pellets Containing 20 mg Riboflavin (with 20 mg Cholesterol). The figures are for gamma of riboflavin per liter urine.

Days before implantation	Rat No. 1	Rat No. 2	Rat No. 3	Rat No. 4	Rat No. 5
14	115	90	95	100	90
12	120	150	100	110	100
10	110	170	110	90	95
8	90	140	90	120	110
6	100	100	75	80	115
4	115	70	90	90	110
2	95	130	110	100	95
Days after implantation					
2	500	500	530	800	600
4	360	300	960	600	700
6	290	290	510	500	860
8	240	250	450	420	510
10	500	300	550	640	400
12	600	900	300	410	350
14	400	800	420	640	280
18	600	480	450	800	700
21	650	320	400	700	840
28	600	200	200	600	600
35	125	260	140	400	510
42		225	110	120	90
49		280		110	
56		170		90	
63		80		100	

woman was implanted with riboflavin pellets (4×25 mg) containing 50 mg riboflavin in all. The riboflavin excretion in a 24-hour specimen of urine was determined (Table II) at 2-day intervals for a period of 2 weeks before the implantation and was found to vary between 650 γ -1000 γ . The patient was kept on a normal diet which would have allowed for variations in the daily riboflavin excretion, between 250 γ -1000 γ per liter of urine. A distinct increase in the amount of riboflavin (1200 γ -2800 γ) excreted in this woman's urine persisted for approximately $1\frac{1}{2}$ months following implantation. This result led us to implant riboflavin in a patient suffering from characteristic glossitis, seborrheic facial lesions, and ocular manifestations such as corneal vascularization. Previous treatment of this patient with orally administered riboflavin in daily doses of 20-30 mg was without any effect. These poor therapeutic results were explained by the existence of complete gastric achlorhydria and chronic diarrhea due to amebiasis. Parenteral riboflavin treatment consisting of 5 mg injections thrice daily gave moderately good but temporary results since 2 days after discontinuance of the treatment signs of riboflavin de-

TABLE II.
Riboflavin Excretion in the Urine of a Normal Female Patient (A) and One Pregnant Patient (B) Suffering from Riboflavin Deficiency Before and After Intramuscular Implantation of Pellets Containing 50 mg Riboflavin (with 50 mg Cholesterol). The figures are for gamma of riboflavin per liter urine.

Days before implantation	Patient A	Patient B
14	870	100
12	900	150
10	650	120
8	800	200
6	950	250
4	1000	150
2	780	170
Days after implantation		
2	1200	1300
4	1800	1500
6	2000	1500
8	2400	2500
10	2800	3000
12	2000	3500
14	2600	3000
18	2200	3500
21	2500	3200
28	2000	2000
35	2000	1500
42	1500	1000
49	950	850
56	690	500

ficiency reappeared. Implantation of pellets containing 50 mg of riboflavin gave good results in this patient with complete absence

of deficiency symptoms for 2 months. At the same time the daily urinary excretion of riboflavin increased from the preimplantation level of 100 γ -250 γ to a concentration of 1000 γ -3500 γ per liter (Table II).

Discussion. The fact that it is possible to maintain a high riboflavin level within the body of animals and man, and to eliminate deficiency symptoms for a period of 1-1½ months, is encouraging. Cases are frequently encountered where regular oral intake of the required riboflavin is ineffective because of inadequate resorption in patients with gastric, intestinal or hepatic diseases. In such patients the riboflavin is only utilized when injected⁶ in repeated daily doses. Since the

maintenance of such treatment is often difficult, if not impossible, we believe that the implantation of pellets has a definite practical value. Furthermore, we intend to apply this method to other vitamins.

Summary. 1. A method is described for the preparation of riboflavin pellets that can be implanted in animals and in humans.

2. The effect of these riboflavin pellets (containing cholesterol) in cases of retarded riboflavin resorption lasts from 1-1½ months.

3. The riboflavin pellets implanted in a patient suffering from riboflavin deficiency brought about complete relief of relatively long duration from deficiency symptoms.

4. The method may be of practical value for patients suffering from riboflavin deficiency due to inadequate resorption, and when repeated injections are difficult to carry out.

⁶ Bicknell, F., and Prescott, F., *The Vitamins in Medicine*, London, W. Heinemann, Ltd., 2nd edit., 1946.

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Action of Sodium Salicylate on Hyaluronidase.

ALBERT DORFMAN, ELIZABETH J. REIMERS, AND MELVIN L. OTT.

From the Division of Chemistry and Physics, Army Medical School, Washington, D.C.

The purpose of this communication is to report certain experiments on the inhibition of hyaluronidase by sodium salicylate *in vivo* and *in vitro*.

Despite wide therapeutic usefulness little is known concerning the mechanism of action of salicylates. A number of investigators have shown that sodium salicylate at relatively high concentrations (0.05 *M*-0.5 *M*), causes a reversible denaturation of certain biologically active proteins.¹⁻⁴ Euler and co-

workers⁵ found that salicylates inhibit certain enzyme reactions in concentrations as low as 0.01 *M*. Some of these are partially reversible by diphosphopyridine nucleotide. Ivanovics⁶ observed that the growth of certain organisms such as *Staphylococcus aureus* was inhibited by concentrations as low as 0.002 *M* while others such as *Proteus morgani* required much larger amounts. He correlated this difference with the ability to synthesize pantothenic acid and found that added pantothenic acid or to a lesser extent β,β -dimethyl- α -hydroxy- γ -butyrolactone was able partially to reverse the salicylate inhibition of *S. aureus*. He concluded that sodium salicylate owes its antibacterial effects to 2 different properties, namely specific inhibition of the synthesis of β,β -dimethyl- α -hydroxy- γ -butyrolactone and nonspecific protein denaturation.

¹ Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, 1934, **17**, 399.

² Holden, H. F., *Austral. J. Exp. Biol. Med.*, 1937, **15**, 43.

³ Bawden, F. C., and Pirie, N. W., *Biochem. J.*, 1940, **34**, 1278.

⁴ Best, R. J., *Austral. J. Exp. Biol. Med.*, 1946, **24**, 27.

⁵ Euler, H. v. and Ahlstrom, L., *Z. physiol. Chem.*, 1943, **270**, 175.

⁶ Ivanovics, G., *Z. physiol. Chem.*, 1942, **270**, 33.

Guerra^{7,8} has recently reported that the intravenous injection of sodium salicylate causes an inhibition of the "spreading effect" of testicular extracts (hyaluronidase) in albino rabbits, as well as in patients either suffering from or having recovered from rheumatic fever. He has concluded that hyaluronidase is inhibited by sodium salicylate and that this activity is responsible for the therapeutic efficacy of this drug in the rheumatic state. This observation seems to be of the utmost importance since one of the most definite facts about rheumatic fever is the efficacy of salicylates in relieving certain symptoms of this disease. The further facts that pathologically this disease may be characterized as a disease of connective tissue and that the enzyme hyaluronidase attacks hyaluronic acid, a component of connective tissue, suggest a possible ray of light in the study of this enigmatic disease.

Duran-Reynals⁹ has pointed out that many factors such as nutrition, age, and sex hormones influence the activity of hyaluronidase *in vivo*. It thus seemed of importance to determine whether sodium salicylate was actually an inhibitor of this enzyme or whether the effects observed by Guerra were due indirectly to changes produced in the animal. Consequently, after verifying the results of Guerra *in vivo*, we have conducted a series of experiments on the effect of sodium salicylate on the activity of hyaluronidase *in vitro*.

Testicular hyaluronidase was prepared from bull testis by the method of Hahn,¹⁰ the purification being carried through the first ammonium sulfate fractionation. Our preparations had an activity of 145 viscosity-reducing units per mg N. Hyaluronidase from *Cl. perfringens* (NIH SR-12) was obtained by growing the organism according to the method of Rogers,¹¹ in a semisynthetic medium containing hyaluronic acid. The

bacterial filtrate was dialyzed at 4°C previous to use. Such preparations had an activity of 500 viscosity reducing units per mg N. Hyaluronic acid was prepared from umbilical cords by a modification of the method of Haas,¹² using a much shorter extraction procedure in the Waring blender.

Spreading activity was determined by injection into the skin of the back of albino rabbits, 0.25 cc of enzyme preparation mixed with 0.25 cc of unfiltered india ink (diluted 1:1). After 24 hours the animal was sacrificed and skinned. The area of spread on the inner surface of the skin was traced on to cellophane and measured with a planimeter. This area was compared to a control area in which india ink mixed with 0.85% saline was injected. This method allows a somewhat more accurate estimation of spreading than has been possible heretofore. Viscosity-reducing activity was measured essentially according to Haas,¹² and was expressed as the reciprocal of the half-life time $\times 10^3$. It was found that when viscosity was plotted against the square root of the time a straight line function was obtained thus making the graphic determination of the half-life time more precise. All assays were done at 37°C. The mucin clot prevention test was found inapplicable to the salicylate experiments since the mixture of sodium salicylate with the horse serum albumin resulted in precipitation. The hydrolysis of hyaluronic acid was followed by the determination of N-acetyl-glucosamine by a modification of the method of Morgan and Elson¹³ utilizing the Colman Junior Spectrophotometer at a wave length of 540 m μ . Salicylates were determined by the method of Brodie.¹⁴

The first experiments conducted were a repetition of those of Guerra using hyaluronidase prepared from *Cl. perfringens* cultures. Sodium salicylate was given both by mouth and intravenously in doses suffi-

⁷ Guerra, F., *Science*, 1946, **103**, 686.

⁸ Guerra, F., *J. Pharm. Exp. Therap.*, 1946, **87**, 143.

⁹ Duran-Reynals, F., *Bact. Rev.*, 1942, **6**, 197.

¹⁰ Hahn, L., *Arkiv. Kemi. Mineral. Geol.*, 1945,

21A, No. 1.

¹¹ Rogers, H. J., *Biochem. J.*, 1945, **39**, 435.

¹² Haas, E., *J. Biol. Chem.*, 1946, **163**, 63.

¹³ Morgan, W. T. J., and Elson, L. A., *Biochem. J.*, 1934, **28**, 988.

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Enzyme incubated with varying concentrations of sodium salicylate for 15 minutes and activity determined by viscosity reducing method. Inhibition is calculated by comparison with control under identical conditions of pH and salt concentration. (pH = 5.8.)

Enzyme	Concentration of sodium salicylate				
	0.20 M	0.10 M	0.05 M	0.025 M	0.013 M
Testicular	100	90	50	42	17
<i>Cl. perfringens</i>	100	75	43	19	0

TABLE II.

Release of N-Acetyl-Glucosamine by Hyaluronidase in Presence of Sodium Salicylate.

Enzyme incubated with hyaluronic acid (12 cc) in 0.2 M acetate buffer at pH 5.0.

Time (hr)	Control mg	Testicular Hyaluronidase.							
		0.12 M		0.09 M		0.06 M		0.03 M	
		*mg	†%	mg	%	mg	%	mg	%
1	300	55	82	120	60	185	38	235	22
2	330	55	84	130	60	230	30	240	27
4	400	55	86	175	54	260	35	320	20
<i>Cl. perfringens</i> Hyaluronidase									
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cient to attain a blood level of 20-30 mg per 100 cc of blood. Definite inhibition of spreading activity of hyaluronidase was obtained in the animals receiving salicylates. These results were similar to those reported by Guerra.

The next group of experiments was performed to determine the effect of sodium salicylate, *in vitro*, on both hyaluronidase prepared from *Cl. perfringens* filtrates and from bull testis. Graded amounts of salicylates were incubated with enzyme and at indicated times samples were withdrawn and assayed. Salicylates were added to the assay mixture in sufficient concentration to maintain the salicylate concentration constant. Total salt and hydrogen ion concentrations were kept constant.

Table I shows the composite results of a group of experiments in which activity was determined by the viscosity reduction method. It is evident that marked inhibition is obtained with a concentration of 0.05 M with both types of enzyme, but at lower concentrations only testicular enzyme was inhibited.

The inhibition of hyaluronidase by salicylates is reversible since removal of salicylate by dialysis produced almost complete restoration of activity.

The effect of salicylates on hyaluronidase was studied further by determining the effect of sodium salicylate on the liberation of N-acetyl-glucosamine. Enzyme was mixed with varying concentrations of sodium salicylate and hyaluronic acid and at definite intervals samples were withdrawn and N-acetyl-glucosamine was determined. Table II illustrates the results obtained under these conditions. It will be noted that a definite inhibition of release of N-acetyl-glucosamine from hyaluronic acid by both testicular and *perfringens* hyaluronidase is obtained. The degree of inhibition is apparently independent of time. Similar results were obtained with the viscosity reduction method. The inhibition of testicular enzyme has consistently been more complete and occurs at lower concentrations. This, it will be noted, is in conformity with the results obtained when the viscosity reduction assay has been used.

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This observation is of interest in view of the recent reports of Haas¹⁰ showing that hyaluronidase from different sources shows different sensitivity to antiinvasin, the hyaluronidase inhibitor present in normal blood. He has attributed these differences to the presence of other substances which he has termed proinvasin I and antiinvasin II. It is possible that our results depend on differences in purity of the various enzymes or merely chemical differences in the hyaluronidases from different sources. That such chemical differences exist is indicated by the work of Hahn.^{10,15}

It is apparent from these data that sodium salicylate does exert an inhibitory effect on the activity of partially purified testicular and perfringens hyaluronidase. The specificity of this effect may be open to doubt in that the concentrations necessary are in the range in which sodium salicylate produces denaturation of other biologically active pro-

teins. This concentration (0.01 *M*-0.1 *M*) is considerably above that obtained therapeutically (0.001 *M*-0.005 *M*). Our work and that of Guerra indicates that sodium salicylate at therapeutic levels does however affect the spreading activity of hyaluronidase. The direct inhibition of hyaluronidase at these concentrations would seem unlikely on the basis of the data presented above but cannot be ruled out in view of the impurity of the preparations used and the variability of sensitivity of hyaluronidases from different sources. The complex mechanism proposed by Haas¹² as well as the demonstration of the role of salicylates in inhibiting antibody formation¹⁰ raise other possibilities as to the locus of action of salicylates on the hyaluronidase system.

Summary. Sodium salicylate has been found to inhibit the spreading effect of hyaluronidase *in vivo*. Hyaluronidase derived from *Cl. perfringens* and bull testis was also inhibited by sodium salicylate *in vitro*. The concentrations necessary for *in vitro* inhibition are considerably higher than those obtained *in vivo*.

¹⁵ Hahn, L., *Arkiv. Kemi. Mineral. Geol.*, 1945, 19A, No. 33.

¹⁶ Homburger, F., *Proc. Soc. Exp. Biol. and Med.*, 1946, 61, 101.

15793

Recovery of Herpes Simplex Virus from Rabbit Brain Nine Months After Inoculation.*

ROBERT A. GOOD.

From the Department of Anatomy, University of Minnesota Medical School, Minneapolis.

We reported the precipitating effect of anaphylactic shock on latent Herpes encephalomyelitis.¹ Further progress has been made in substantiating our original observations.² Although at that time we did not demonstrate the existence of the Herpes simplex virus in the central nervous tissues of the rabbits showing the encephalitic symp-

toms following anaphylactic shock, we pointed out the symptomatological and pathological identity of the disease produced with that of Herpes encephalomyelitis. We further reported the finding of intranuclear inclusion bodies in the brain of the rabbit. Experiments pointing out the production of encephalitic symptomatology and central nervous system pathology by various noninfective mechanisms³⁻⁶ made it of considera-

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

¹ Good, R. A., and Campbell, B., *Proc. Soc. Exp. Biol. and Med.*, 1945, 59, 305.

² Campbell, B., and Good, R. A., unpublished MS.

³ Rivers, T. M., and Schwenkter, F. F., *J. Exp. Med.*, 1935, 61, 689.

⁴ Ferraro, A., *Arch. Neurol. and Psychiat.*, 1944, 52, 443.

Protocol—Rabbit G 370.

11/8	One young adult albino rabbit was sensitized to egg white by administration of the following dosages on alternate days: 1 cc I.V.; .5 cc I.V.; and 1 cc I.M.
11/12/45	
12/27	The rabbit was inoculated in the quadriceps muscle with 1 cc of a 10 ⁻¹ suspension of 11. F. ₂₁ virus.
1/6/46	Definite evidence of encephalomyelitis, elevated temperature, paralysis of the right hind leg and encephalitic manifestations.
1/18	Temperature normal, no evidence of encephalitis, no paralysis. Apparent recovery from a mild Herpetic encephalomyelitis.
1/20	After temperature had been normal for 11 days and there was no evidence of encephalitis or myelitis the animal exhibited moderate anaphylactic shock following intravenous administration of .15 cc egg white.
2/7	Slight elevation of rectal temperature.
2/7	Development of full blown encephalomyelitis. The symptoms and signs included elevated temperature to 105.5, paralysis of both hind legs, weakness of front legs, excessive salivation, opisthotony, nystagmus, and facial twitching.
2/27	Temperature returned to normal and except for a few sequelae, animal essentially normal.
2/27	Temperature remained normal.
4/15	
4/15	Mild anaphylactic shock.
4/22	Temperature elevated, nystagmus, increased salivation, gnashing of teeth and disorientation.
4/22	Elevated temperature and evidence of mild but definite encephalitis.
5/1	
5/1	Essentially normal except for slight disposture of the head.
7/10	
7/10	Severe anaphylactic shock produced by the intravenous administration of .3 cc egg white I.V.
7/18	Temperature elevation noted, animal began to appear ill.
7/18	Gradual progressive encephalomyelitis with very high fever and severe symptomatology.
8/30	
8/30	Dead.

ble importance to know whether or not an active virus agent is present in the nervous tissues of the animals suffering acute exacerbations of encephalitis. Hurst, Cooke, and Melvin⁷ reported the isolation of canine distemper virus from the central nervous sys-

tem of dogs 6 weeks after a systemic distemper infection at a time when the dogs showed symptoms of central nervous system involvement. Brodie⁸ and Bodian and Cumberland⁹ have reported the recovery of poliomyelitis virus from the central nervous system of monkeys 16 and 14 days respectively after disappearance of the paralysis. The persistent excretion of polio virus in the stools of convalescents, Horstman and Melnick,¹⁰ has received considerable attention.

This protocol illustrates the capacity of anaphylactic shock to precipitate latent virus encephalitis since on all 3 occasions when the animal was shocked symptoms and signs of encephalitis followed after a latent period of one week.

Recovery and Identification of the Virus.

Under aseptic precautions samples of central nervous tissue were removed from the lumbar spinal cord, medulla oblongata, cerebellum, cerebral cortex and thalamus of Rabbit 370 and placed in separate test tubes containing 50% buffered glycerine solution (pH 7.4). After 2 days' storage at ice box temperature each sample of tissue was ground in a sterile mortar and suspended in saline to form a 1:10 dilution of rabbit brain.[†]

Three mice were inoculated with .03 cc of the tissue suspension from each of the above described areas of the rabbit's central nervous system. All the mice died within 3 days except those inoculated with the medulla oblongata tissue. On the third intracranial passage in mice a 10⁻¹ suspension of the virus killed all mice inoculated on the second day after inoculation. A determination of the M.L.D.₁₀₀ showed it be .03 cc of a 1/10,000 dilution of mouse brain in normal saline.[‡]

⁸ Brodie, Proc. Soc. Exp. Biol. and Med., 1933, 30, 1238.

⁹ Bodian, D., and Cumberland, *Am. J. Hyg.*, 1947, in press.

¹⁰ Horstman and Melnick, *J. A. M. A.*, 1944, 126, 1061.

[†] These suspensions showed no evidence of bacterial growth on blood agar streak plates or Brewer's medium deep cultures.

[‡] This was the same M.L.D.₁₀₀ as the H.F.₂₁ virus possessed which was inoculated into G 370 nine months previously.

⁵ Kabat, E. A., Wolf, A., and Bezer, A. E., *Science*, 1946, 104, 362.

⁶ Morgan, I., *J. Exp. Med.*, 1946, 85, 131.

⁷ Hurst, E. W., Cooke, B. T., and Melvin, P., *Aust. J. Exp. Biol. and Med. Science*, 1943, 21, 115.

The virus, after a subsequent passage in mice, was inoculated onto the cornea of a young adult rabbit. This rabbit developed a kerrato-conjunctivitis typical of Herpes infection.[§] After a period of 8 days this rabbit developed a fulminating encephalitis and died. Pathological study of the brain revealed findings typical of Herpetic encephalitis.

In another rabbit, No. 548, following the precipitation of active encephalomyelitis from the latent state by anaphylactic shock 120 days after inoculation of Herpes simplex virus into the quadriceps femoris muscle

§ Corneal serapings were obtained from the infected eye three days after inoculation and showed typical intranuclear inclusion bodies in the epithelial cells.

group, a virus agent was again recovered from the central nervous tissues. The technics of recovery of the virus and its identification by biological characteristics were the same as were used in Rabbit No. 370. The virus after 3 mouse passages was identical in all respects to the virus inoculated 4 months previously.

Summary. 1. An infectious agent was recovered from the central nervous system of a rabbit which had been inoculated 9 months previously with Herpes simplex virus and which had suffered 3 acute relapses of the disease. The virus recovered exhibited characteristics identical with those of the original virus. 2. A second instance of virus recovery 4 months after inoculation is reported.

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Artifacts in Gold Shadowed Electron Micrographs Due to Electrons of High Intensity.

R. J. MANDLE. (Introduced by W. M. Stanley.)

From the Department of Animal and Plant Pathology, The Rockefeller Institute for Medical Research, Princeton, N.J.

The electron microscope has been used widely for the examination of virus preparations, for the sizes of many viruses are below the range in which the light microscope can be used to good advantage.¹ However, because of the low contrast which is afforded by small viruses, difficulties in securing good electron micrographs have been encountered. Electron microscope studies on small viruses were aided immensely by the development of the metal shadow-casting technic by Williams and Wyckoff.^{2,3} This technic has also proved of value in securing good definition of virus particles in the presence of a background of material of smaller particle size.

By means of this technic Sigurgeirsson and Stanley⁴ and Oster and Stanley⁵ have secured good delineation of the particles of tobacco mosaic virus in the unpurified juice and contents of hair cells, respectively, of plants diseased with tobacco mosaic. Although there was no difficulty in these studies in showing that most of the rods present in fresh virus preparations possessed a length of 280 m μ , occasional pictures were obtained which showed segmentation of the virus rods similar to that described by Williams and Wyckoff.⁶ Further study has shown that this segmentation into shorter pieces of variable

⁴ Sigurgeirsson, T., and Stanley, W. M., *Phytopathology*, 1947, **37**, 26.

⁵ Oster, G., and Stanley, W. M., *Brit. J. Exp. Path.*, 1946, **27**, 261.

⁶ Williams, R. C., and Wyckoff, R. W. G., *Science*, 1945, **101**, 594.

¹ Stanley, W. M., *Chronica Botanica*, 1943, **7**, 291.

² Williams, R. C., and Wyckoff, R. W. G., *J. Applied Physics*, 1944, **15**, 712.

³ Williams, R. C., and Wyckoff, R. W. G., *Proc. Soc. Exp. Biol. and Med.*, 1945, **58**, 265.

lengths is due to an artifact. This artifact and its controlled formation are described in the present paper.

Methods. Tobacco mosaic virus from infected Turkish tobacco plants, isolated and purified by differential centrifugation,⁷ was prepared in a suitable dilution and placed on the copper screens covered with a thin film of collodion in the normal fashion of preparing specimens for use in the electron microscope. The screens were then subjected to the oblique deposition of a very thin evaporated metal film after the manner described by Williams and Wyckoff. The apparatus used in this laboratory consists of a glass chamber mounted on a polished brass plate in which there are 4 supports for the 1 × 3 glass slides which carry the mounts. The chamber is evacuated to less than 10⁻⁵ mm. of mercury. The gold used for the shadow casting was deposited from a hot tungsten filament 20 cm. above and at an angle of 15° to the glass slides. The amount of gold deposited was controlled by observing the color of the light transmitted through the slide and comparing it to a sheltered "standard" slide placed within the chamber. Since the slides were arranged with their long axis toward the gold source, a gradation of gold was obtainable on each slide so that it was possible to select the screen with the desired amount of deposited gold.

An RCA Console Model (type EMC-1) electron microscope yielding a magnification of 5800 diameters was used for the observation of the specimens. In order to obtain the initial electron micrographs the minimum intensity that would still allow accurate focusing of the image on the fluorescent screen was used. After a suitable field had been procured, a micrograph was taken with as little delay as possible. The exact field was maintained and the intensity was raised to the maximum. Following the exposure of the specimen to the high intensity electron beam for such time as was necessary to secure the desired effect, usually about 5 minutes, a micrograph was again taken. In this manner it became possible to compare images

of identical virus particles following exposure to low and to high intensity electron beams.

Discussion of Results. Fig. 1 shows the virus particles as they appear in a micrograph taken at low intensity. The background is uniform and the particles appear well defined. Fig. 2 shows the same particles after they had been subjected to the high intensity beam for 3 minutes. The background has become very granular and the virus particles appear segmented. This segmentation appears to be similar to that described by Williams and Wyckoff.⁶

Fig. 3 and 4 show another set of micrographs treated in a similar manner except that Fig. 4 was taken after 10 minutes of intense irradiation. It can be seen that, although the background has become quite granular, the degree of segmentation appears to be considerably less than in Fig. 2. Continued irradiation did not produce the extreme segmentation noted in Fig. 1. This may have been due to a difference in the thickness of the gold deposited on the surface of the particles. It can also be seen from a comparison of Fig. 3 and 4 that there are many particles missing or obscured in Fig. 4. The letters correspond to the same particle or group in each figure. There appears to be some correlation between the direction of the deposition of the gold in relation to the orientation of the particle and the obscuring of the particle.

Fig. 5 shows an extreme effect which can result from prolonged exposure at the maximum intensity. Some of the particles appear segmented and others show only a single very dense circular area. It seems likely that on prolonged exposure to a high intensity electron beam the gold becomes fluid and forms globules on the surface of the virus particle and on the collodion membrane. Since the gold globules may coalesce to different sizes, the resulting appearance may then vary from the smallest ones, giving the effect of segmentation of the particle, to the larger globules which are shown in Fig. 5. The fine beading or segmentation shown in Fig. 2 could easily be mistaken as evidence for the existence of small segments of the tobacco

⁷ Stanley, W. M., *J. Biol. Chem.*, 1940, **135**, 437.



FIG. 1. Electron micrograph taken at low intensity of tobacco mosaic virus prepared with gold by the shadow-casting technique.

FIG. 2. The same specimen as that shown in Fig. 1 following exposure to a high intensity electron beam for 3 minutes. Many of the virus rods appear segmented.

mosaic virus rod. However it is obvious from Fig. 1 that the appearance of segmentation

in Fig. 2 is an artifact resulting from exposure to the electron beam. It seems likely



FIG. 3. Electron micrograph taken at low intensity of tobacco mosaic virus prepared with gold by the shadow-casting technique. Letters indicate identical rods in Fig. 4.

FIG. 4. The same specimen as that shown in Fig. 3 following exposure to a high intensity electron beam for 10 minutes. The exposure has caused the background to become granular and several of the particles are missing or not well defined.

FIG. 5. Tobacco mosaic virus prepared with gold by the shadow-casting technique after prolonged exposure to an electron beam at high intensity. The picture shows an extreme case of segmentation or bead formation.

that the segmentation of tobacco mosaic virus described by Williams and Wyckoff⁶ was caused in a similar manner.

An effort was made to ascertain whether or not segmentation could be produced in tobacco mosaic virus preparations before shadow casting with gold or in preparations stained with phosphotungstic acid. No evidence for segmentation following prolonged exposure to a high intensity electron beam was obtained in either case. The results confirm the statement by Stanley and Anderson⁸ for unshadowed material that "The fact that a micrograph taken with the first flow of electrons through a specimen does not appear to differ from subsequent micrographs taken after longer exposure to the electron beam makes it seem unlikely that gross changes are caused by the electrons."

The results of the present study indicate that in order to reduce or eliminate granulation and other obvious artifacts, which can

occur when the shadow-casting technic with gold is used, it is necessary to avoid prolonged exposure of the specimen to the intense electron beam.

Summary. Electron micrographs of gold shadow-cast specimens of tobacco mosaic virus show that artifacts, consisting of segmented or even globular particles, are formed when the specimen is exposed to the beam of electrons at high intensity. It is suggested that these are caused by the thin film of gold becoming fluid and forming globules on the surface of the virus rods and supporting membrane. Tobacco mosaic virus not treated with gold did not show any change after having been subjected to the same treatment. The results indicate that gold covered specimens should not be subjected to prolonged exposure to an electron beam of high intensity.

It is a pleasure to acknowledge my indebtedness to Dr. W. M. Stanley for his active interest and helpful guidance.

⁸ Stanley, W. M., and Anderson, T. F., *J. Biol. Chem.*, 1941, **139**, 325.

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Hypersensitivity to Egg White in the Rat.*

JACQUES LÉGER,[†] GEORGES MASSON, AND J. LEAL PRADO.[‡] (Introduced by Hans Selye.)

From the Institut de Médecine et de Chirurgie Expérimentales, Université de Montréal, Montréal, Canada.

Injection of egg white into previously sensitized rats elicits symptoms which have been taken as proof of an anaphylactic reaction.^{1,2} Among these symptoms are hyperemia, cyanosis and edema. One author¹ mentioned that egg white has no primary toxicity.

* These investigations were aided by a grant made by The Sugar Research Foundation to Dr. H. Selye.

[†] Fellow, National Research Council of Canada, Division of Medical Sciences.

[‡] Fellow, Canada-Brazil Trust Fund, Instituto Butantan, Sao Paulo, Brazil.

¹ Molomut, N., *J. Immunology*, 1939, **37**, 113.

² Pratt, H. N., *J. Immunology*, 1935, **29**, 301.

On the other hand it has been reported³ that egg white administered parenterally is toxic and that sensitized and unsensitized rats reacted with almost identical symptoms. Among the symptoms there is a marked edema of the face, tongue, clitoris and paws preceded by hyperemia.⁴ It seems therefore that the rat is naturally hypersensitive to egg white and that the symptoms attributed to anaphylaxis may be the result of this hypersensitivity. Since hypersensitivity is very seldom observed in animals from the first

³ Parker, J. T., and Parker, F., Jr., *J. Med. Res.*, 1924, **44**, 263.

⁴ Selye, H., *Endocrinology*, 1937, **21**, 169.

contact, this reaction offered an opportunity to approach it experimentally. The purpose of the present investigation is mainly to standardize the conditions under which this reaction will be further studied.

Experimental. In a preliminary experiment 12 male, hooded rats weighing 150-200 g were injected intraperitoneally with a dose of 3 cc of fresh egg white. The animals were examined 30 minutes, 2, 4 and 23 hours after the injection in order to determine when the reaction reaches its maximum. The only symptoms observed consisted of edema of the face, tongue and paws which was preceded by hyperemia noticeable mainly on the nose, ears and paws. The intensity of the edema, read separately for the face and paws, was recorded according to a scale from 0 to 3: 0, no reaction; 1, slight but definite reaction; 2, moderate to marked reaction and 3, very marked reaction. The results were as follows: after 30', incidence 15%, severity 12%; after 2 hours, incidence 100%, severity 84%; after 4 hours, incidence 100%, severity 70%; after 23 hours no more edema was visible. We therefore decided, in any further experiments, to examine the animals 2 hours after an intraperitoneal injection of egg white.

When the egg white is injected intravenously the symptoms appear more rapidly. Within a few minutes following injection there is hyperemia, followed by edema, which is maximum after 10 to 30 minutes. In some animals we noticed a cyanosis of the extremities, especially of the paws; in these cases there was no subsequent edema and the animals seemed otherwise normal.

It must be pointed out that this reaction does not apply to all proteins, because the injection of large doses of horse serum was without effect.

Having established that the rat is hypersensitive to egg white we devised another experiment in order to see whether any relationship existed between the dose of egg white injected and the incidence and intensity of the symptoms. Male albino rats weighing 60-80 g were divided into 6 groups of at least 10 animals each. Each group received

TABLE I.
Effect of Dose of Egg White on Incidence and Intensity of the Edema.

Doses in cc	No. of animals	% incidence	% intensity
1.00	9	89	61
0.50	10	100	62
0.25	10	90	45
0.20	10	70	40
0.15	40	57	33
0.10	10	25	9

intraperitoneally one injection of 1 cc, 0.5 cc, 0.25 cc, 0.15 cc and 0.10 cc of fresh egg white respectively. The animals were examined after 2 hours; the results are summarized in Table I.

There is, evidently, a direct relationship between the dose and the response, which is more definite in regard to the incidence than to the intensity. The dose which gives approximately 50% of response is 0.15 cc.

In order to determine the variability of the response in the same animal and to see whether there is acquired tolerance, the animals of the latter experiment were injected, during 9 consecutive days, with the same initial dose of egg white. The responses were read 2 hours later. Since all the animals behaved similarly, we list in Table II only the observations on the first 4 animals of the groups receiving 1 cc, 0.5 cc and 0.10 cc of egg white.

From the results summarized in Table II it can be seen that: (1) the same intensity of edema is not necessarily present at the same time on the face and the paws or vice-versa; one animal may have a reaction of 3 on the face and no edema on the paws. There is no predilection for one area rather than for another. (2) The sensitivity of the animals varies from day to day. (3) On continuous treatment the sensitivity does not necessarily disappear.

Among the other variables, the composition of egg white should be taken into consideration. To eliminate this factor, we lyophilized a large quantity of egg white using it as a uniform stock for all these experiments.

It is known that age and sex have an influence on certain biological reactions. To in-

that the segmentation of tobacco mosaic virus described by Williams and Wyckoff⁶ was caused in a similar manner.

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⁸ Stanley, W. M., and Anderson, T. F., *J. Biol. Chem.*, 1941, **139**, 325.

15795

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On the other hand it has been reported³ that egg white administered parenterally is toxic and that sensitized and unsensitized rats reacted with almost identical symptoms. Among the symptoms there is a marked edema of the face, tongue, clitoris and paws preceded by hyperemia.⁴ It seems therefore that the rat is naturally hypersensitive to egg white and that the symptoms attributed to anaphylaxis may be the result of this hypersensitivity. Since hypersensitivity is very seldom observed in animals from the first

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¹ Molomut, N., *J. Immunology*, 1939, **37**, 113.

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³ Parker, J. T., and Parker, F., Jr., *J. Med. Res.*, 1924, **44**, 263.

⁴ Selye, H., *Endocrinology*, 1937, **21**, 169.



FIG. 1.
Normal control rat.

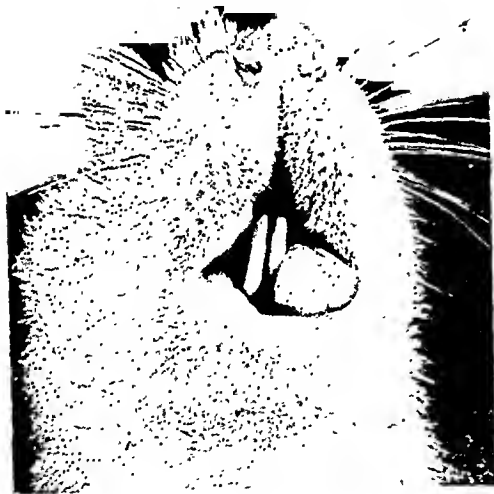


FIG. 2.
Egg-white treated rat. Note edema of tongue, nose and face. The two upper incisors are not visible because of edema and also the medial line of tongue is not as defined as in the control.

One author has already attributed the symptoms following parenteral injection of egg white to the toxicity of the substance.³ Such a possibility is suggested by the relationship existing between dose and response. Against such an interpretation are the facts that even with high doses of egg white the symptoms are always mild and that the incidence and the severity of the reaction vary



FIG. 3.
Left, paw of normal control rat. Right, edema of the paw after injection of egg white.

from day to day.

The reaction has some similarity with the local anaphylaxis and the symptoms elicited by the local injection or release of histamine. Against the anaphylactic theory is the fact that there is no need for a previous sensitization of the animals, and therefore no explanation can be based on an antigen-antibody mechanism. It seems to the authors that, tentatively, the egg-white reaction can be classified as an anaphylactoid phenomenon which is defined as a group of reactions resembling in their symptomatology those of true anaphylaxis but which cannot be referred to a specific antigen-antibody mechanism.⁵ The inhibition of the egg-white reaction by antihistaminic substances gives some support to this interpretation.⁶

Although anaphylaxis is very difficult to elicit in the rat, the reaction to egg white can readily be obtained in a high percentage of the animals. It can be assumed therefore that different mechanisms are involved.

The symptoms obtained with egg white

⁵ Zinsser, H., Enders, J. F., and Fothergill, L. D., *Immunity. Principles and Application in Medicine and Public Health*, The Macmillan Co., New York, 1941.

⁶ Leger, J., and Masson, G., in press.

TABLE II.
Influence of Dose and Duration of Treatment on Production of Edema.

Group	Daily dose, cc	Rat	Day 1st	2nd	3rd	5th	6th	7th	8th	9th
I	1	A	3;3	0;0	2;2	2;0	0;1	0;0	0;0	0;0
		B	3;3	0;0	1;2	0;0	0;1	0;0	0;0	0;0
		C	0;0	1;0	0;0	0;0	0;0	0;0	0;0	0;0
		D	2;2	1;0	0;0	0;3	0;1	1;2	—	0;1
II	0.5	A	2;2	3;3	0;0	0;3	0;2	0;1	1;0	1;2
		B	1;2	0;1	0;0	1;3	0;0	1;0	0;0	0;0
		C	2;3	1;1	0;0	2;2	0;0	0;1	0;0	0;0
		D	3;3	1;1	0;0	3;2	1;0	0;0	0;0	0;0
III	0.25	A	3;2	0;1	0;0	1;0	0;2	0;1	0;0	0;1
		B	1;1	2;2	0;2	0;0	0;1	1;3	0;0	2;2
		C	3;3	0;0	1;0	2;0	0;0	0;0	0;0	1;2
		D	3;3	0;0	0;0	3;3	0;0	0;0	0;2	2;2
IV	0.10	A	0;0	0;0	0;0	0;0	0;0	0;0	1;1	0;0
		B	1;0	0;0	0;0	0;0	0;0	0;0	0;0	0;0
		C	0;0	0;0	0;0	2;0	0;0	1;3	0;0	0;0
		D	1;0	1;1	0;0	0;0	0;0	0;0	0;0	1;2

The first number in each column represents the intensity of edema on the face, the second on the paws.

TABLE III.
Influence of Age and Sex on Production of Edema.

				1st day		4th day		6th day	
				% incidence	% intensity	% incidence	% intensity	% incidence	% intensity
Group I	♂	40-50 g		89	37	25	12	75	33
Group II	♂	70-80 g		62	25	17	3	20	3
Group III	♂	120-140 g		75	37	12	4	29	7
Group IV	♀	120-140 g		37	19	12	4	25	11

investigate this possibility we performed the following experiment. White rats were divided into 4 groups of 8 animals: Group I, male rats weighing 40-50 g; Group II, male rats weighing 70-80 g; Group III, male rats weighing 120-140 g, and Group IV, female rats weighing 120-140 g. All the animals received one intraperitoneal injection of 0.15 cc of egg white on the 1st, 4th and 6th day. The results are summarized in Table III.

Age evidently does not influence markedly the sensitivity of the animals; as far as sex differences are concerned it seems that the females are somewhat less sensitive although experiments with larger groups of animals would be necessary to provide definite proof of this point.

During the course of the present investigation we noticed that a few animals presented disturbances in the vestibular equilibrium apparatus resembling those seen in the

Ménière's syndrome. Since they appear only after injection of egg white, the possibility of spontaneous otitis media can be eliminated.

Discussion. Judged by the present experiments, the egg-white reaction shows the following characteristics: (1) it occurs naturally in rats, (2) it is elicited at the first parenteral injection, (3) there is a direct relationship between dose and incidence or severity of the reaction, (4) the symptoms are always mild and restricted to certain areas: no case of severe reaction or fatal shock has been observed, (5) various areas are not necessarily affected to the same degree, (6) on continuous treatment the sensitivity of the animals varies from day to day; there is no permanent immunity.

It now remains to be determined whether these symptoms should merely be considered as part of a simple toxic reaction or classified among the phenomena of hypersensitiveness.

A Simple Effective Regimen for Preservation of the Dog With a Pancreatic Fistula.

FRED KOLOUCH.*

From the Department of Surgery, University of Minnesota Medical School, Minneapolis.

Dogs tolerate pancreatic fistulas poorly.¹ In addition to the chronic excoriation of the skin of the abdomen and legs, general physical deterioration is rapid. The anorexia, dehydration, hypochloremia, cachexia and ultimate death can be obviated by refeeding pancreatic juice or by parenteral administration of saline in addition to their regular diet.¹⁻³ The technical difficulties attending the maintenance of dogs with pancreatic fistulas is appreciated by anyone who has worked with these animals.

The author was confronted with a need for dogs with simple Pavlov type pancreatic fistulas which would survive for a prolonged period of time at a level of health compatible with the tolerance of major surgery (gastrectomy).⁴ A simple effective maintenance regimen for these animals was developed, which successfully prevented skin excoriation and preserved the general health and vigor of these animals.

The skin of the abdomen and legs was protected from the irritative effects of pancreatic juice by maintaining the animals in trays of clean, fine, white sand. The dogs soon learned to keep their bellies nestled in this absorptive material, thereby obviating skin excoriation. It was necessary to change the sand twice daily.

* Fellow of the National Cancer Institute, Bethesda, Md.

The research presented here was supported by grants of the Graduate School of the University of Minnesota, the John and Mary R. Markle Foundation, the Augustus L. Searle Fund for Surgical Research, and the Citizens' Aid Society.

¹ Elman, R., and McCaughan, J. M., *J. Exp. Med.*, 1927, **45**, 561.

² Cowgill, G. R., *J. Biol. Chem.*, 1923, **56**, 725.

³ Allen, J. S., Vermeulen, C., Owens, F. M., and Dragstedt, L. R., *Am. J. Physiol.*, 1943, **138**, 352.

⁴ Kolouch, Fred, unpublished.

TABLE I.
Daily Ration for Dog (Weight 15 kg) with Pancreatic Fistula.

Constituents	Quantity
Raw pork pancreas	150 g
Beef tallow	40 "
Casein	100 "
Sucrose	100 "
Milk	500 "
Cod liver oil	4 cc
Brewers yeast	6 tablets
Vit. C	25.0 mg
Vit. K	1.0 "
Cowgills (2) salt mixture	4.14 g
Sodium bicarbonate	1.5 "
Bone ash	7.5 "
Amphogel	30.0 cc

Diet homogenized and administered by gastric intubation if necessary.

The daily replacement of the water, electrolytes, and sufficient pancreatic enzymes was met by the forced feeding of a ration of a homogenized liquid diet, nutritionally complete, for a dog with a pancreatic fistula. The dietary formula is listed in Table I. When necessary, in anorexic dogs, tube feeding insured complete ingestion of this diet. Antacids were added to prevent development of peptic ulcer incident to the loss of the normal neutralizing effects of pancreatic juice.²

Maintained on the above regimen, 5 of 6 dogs with pancreatic fistulas survived gastrectomy. The mortality in the 6th dog was due to a surgical blunder. Following gastrectomy, survival varied from 3 months to 2½ years. Deprivation of the special regimen with return to ordinary kennel care and rations evoked a dramatic deterioration and ultimate death in all of these gastrectomized dogs.

Summary. (1) A simple kennel and dietary routine for the maintenance of the health and vigor of dogs with pancreatic fistulas is outlined. (2) Pancreatic fistula dogs supported by this regimen tolerated major abdominal surgery (gastrectomy).

are similar to those described for angioneurotic edema in man: edema of the face, lips, buccal cavity associated with vestibular crisis or other neurologic manifestations. Another similarity is the abrupt appearance of the symptoms and their sudden disappearance.

Summary. In the rat a single intravenous or intraperitoneal injection of egg white elicits a reaction characterized by hyperemia, followed by edema of the face, tongue and paws. Although only these regions respond,

they are not necessarily affected to an equal extent. Continued injections of the same dose produce variable reactions in the same animals but there is no acquired tolerance. Age does not play a great influence, but females seem less sensitive than males.

The authors wish to express their sincere appreciation to Dr. M. Rocha e Silva for suggesting the subject of this investigation and for his helpful advice.

15796 P

Cholinesterase Content of Erythrocytes in Normal and Pathological States. I. Content in Normal Humans.*

JOSEPH K. CLINE, ROBERT BRUCE JOHNSON, AND WALTER HAMILTON JOHNSON.
(Introduced by E. B. Carmichael.)

From the Biochemistry Department, Medical College of Alabama, Birmingham.

Several investigators have recently recently reported changes in plasma, or whole blood cholinesterase content in anemias and leukemia. Values reported were expressed in terms of cholinesterase activity per volume of sample. Sabine¹ has calculated the cholinesterase content per cc of red cells but was unable to secure accurate values by direct measurement.

The red cells contain only specific cholinesterase and none of the nonspecific type found in plasma or serum. It is unlikely, therefore, that the cholinesterase in the red cells is derived from the circulating plasma, but, more probably, their esterase content is produced by the tissues from which they are derived or with which they are closely associated. Any change of esterase content per cell may be important, therefore, in reflecting the activity of the reticulo-endothelial elements of bone-marrow. The production of the esterase by the red cell itself cannot at this time be excluded.

Preliminary to some observations of the red cell content in various pathological states, we have determined the cholinesterase content per million cells in 18 normal human subjects. We have been able to secure accurate values by using volumes of washed, resuspended red cells calculated to contain the same number of cells as determined by cell count. The cells were lysed with 10% NaCl at 2°C. The measurements were then conducted at 37.5° by a modification of the method of Ammon² in which the bicarbonate-Ringers solution and the acetylcholine chloride solution were both placed in the large space and the blood sample in the side cup of the Warburg vessels. Values of cholinesterase activity thus secured are expressed as cmm of CO₂ produced per hour per million red cells. Esterase activity so expressed was not proportional to cell count or total blood hemoglobin. Values for different subjects varied from 3.94×10^{-4} cmm to 7.45×10^{-4} cmm with a mean value for normals of 6.16×10^{-4} cmm.

* Aided by a grant from the Williams-Waterman Fund of Research Corporation.

¹ Sabine, J. C., *J. Clin. Invest.*, 1940, **10**, 833.

² Ammon, R., *Arch. ges. Physiol.*, 1934, **233**, 486.

TABLE I.

Agglutination of Normal Erythrocytes by Anti-human Globulin Serum After Exposure to Eluate from Erythrocytes of a Patient with Acquired Hemolytic Anemia. Positive results were obtained only from suspensions which stood at ice box temperature. Agglutination of samples of the same suspension was increased by subsequent incubation at 37°C.

	Temp. incubation, °C	Agglutination in anti-globulin serum
Eluate from patient's cells	37	0
plus	20	0
0.1 volume normal O cells	5	+ (37°C 2 hr) ++
Eluate from normal cells	37	0
plus	20	0
0.1 volume normal O cells	5	0 (37°C 2 hr) 0

accelerated rate.⁵ A hemolysin is presumed to be the cause of the hemolytic anemia which has continued without improvement. Her erythrocytes have shown a consistent agglutinability in all dilutions of the anti-globulin serum as high as 1-80 in contrast to the absence of agglutination in any dilution of normal erythrocytes or cells from patients with congenital hemolytic jaundice. It was noted in preparation of the eluate that exposure of the patient's cells to 56°C for 5 minutes removed or destroyed this agglutinable property.

When normal group O cells were suspended in the eluates prepared in the manner described above only those cells which had been suspended in the eluate from the patient's cells and kept at ice box temperature showed a tendency to agglutinate in the anti-globulin serum, while the cells suspended in the eluate from the control showed no tendency to agglutinate. Cell suspensions kept at room temperature and 37°C gave negative results. It was observed that agglutinability of normal O cells after exposure to the eluate from the patient's cells became more pronounced if, after removal from the ice box, a sample of the suspension was allowed to stand at 37°C for 1 or 2 hours before the cells were washed and suspended in the anti-globulin serum shown in Table I. These observations were repeated with freshly prepared eluate and cells of various types with the same results.

A second patient with an acute hemolytic anemia of the acquired type was studied

and observations of similar nature have been made. She was 23 years of age and had no past or family history suggestive of hemolytic disease. For 3 weeks she had noted increasing weakness, nausea and yellow color. She was pale and jaundiced and the spleen was palpable below the costal margin. The hematocrit was 10, and Hb. 3.4 g/100 cc. Reticulocytes were 10% and the icterus index 50. Red cell fragility in hypotonic saline was greatly increased and spherocytosis was evident in the blood smear. She was group O, Rh₁. Repeated multiple transfusions had a temporary effect in elevating the erythrocyte count and hemoglobin and it seemed certain without more exact determinations that transfused cells were involved in the hemolytic process. Cold agglutinins were demonstrated in a titer of 1-32 and an atypical agglutinin active at 37°C was demonstrable for about 30% of O cells but had no Rh or MN specificity. An atypical hemolysin was not demonstrated.

This patient's erythrocytes showed marked agglutination in a 1-160 dilution of the anti-globulin rabbit serum even when observations were carried out at 37°C to eliminate the cold agglutinin as a factor. Her cells failed to show agglutination after exposure to 56°C for 5 minutes. Normal O cells from 3 individuals showed no agglutination in her serum, but when incubated in the eluate from her cells acquired a susceptibility to agglutination in the anti-globulin serum as shown in Table II.

Discussion. It is evident that the erythrocytes from the 2 patients with acquired hemolytic anemia had a property uniquely

⁵ Evans, R. S., *Arch. Int. Med.*, 1946, 77, 544.

Demonstration of Antibodies in Acquired Hemolytic Anemia With Anti-Human Globulin Serum.

ROBERT S. EVANS, ROSE T. DUANE, AND VERA BEHRENDT.
(Introduced by A. L. Bloomfield.)

From the Department of Medicine, Stanford University School of Medicine, San Francisco, Calif.

The technic described by Coombs, Mourant and Race¹ for detecting the presence of the heat stable or "blocking" Rh antibody by agglutinating the sensitized cells with an anti-globulin rabbit serum should be applicable to the investigation of other types of hemolytic disease in which the presence of an immune body is suspected. Agglutination of the sensitized cells depends on the interaction of the attached globulin of the blocking antibody and the antiglobulin rabbit antibody. It has been demonstrated by Boorman, Dodd and Loutit² that washed erythrocytes from patients with acquired hemolytic anemia are agglutinated in the antiglobulin rabbit serum whereas those from patients with familial hemolytic jaundice are not, indicating that only the former have a globulin antibody attached to the cell. Observations are reported here which indicate that the erythrocytes of 2 patients with acquired hemolytic anemia were coated with an immune body which could be released and transferred to normal cells *in vitro*.

Methods. The immune rabbit serum was produced by the intracutaneous and subcutaneous injection of 1 cc of group O human serum at weekly intervals for 2 courses of 4 injections each. The cell antibodies in the serum were absorbed by incubation with an equal volume of a 50% saline suspension of pooled A and B cells at 37°C for one hour. After 2 absorptions the serum did not agglutinate normal cells of any group or type, but when further diluted 1-10 with isotonic saline it produced agglutination of Rh₁ cells after they had been exposed to a 1-128 saline dilution of antiRh₀ serum containing

only the heat stable or "blocking" antibody. The agglutinating titer of the antiRh₀ serum when diluted with 30% beef albumin was 1-128. Serial dilutions of the immune rabbit serum produced a visible precipitin reaction with a 1-10,000 dilution of commercial preparation of human immune globulin.

Erythrocytes were obtained by collecting capillary blood in normal saline or by defibrinating venous blood. The cells were washed 3 times with normal saline before suspending in the antiglobulin serum. The capillary tube technic³ as well as the usual test tube method followed by centrifugation were used to detect agglutination and results were checked by observation of the suspensions on a glass slide.

The elution technic of Landsteiner and Miller⁴ was used in the transference of the erythrocyte antibody as follows: Washed cells from the patient and from a normal group O individual were each suspended in twice their volume of normal saline and kept in a 56° water bath for 5 minutes with gentle agitation. The suspensions were then centrifuged quickly in heated cups and the supernatant fluid removed. The degree of hemolysis was observed to be roughly equal in the 2 samples. The eluate was divided into 1 cc amounts and 0.1 cc of a concentrated suspension of normal group O cells was added. The suspensions were kept over night at varying temperatures with intermittent agitation. At the end of this period samples were removed and the cells washed before suspending in the antiglobulin serum.

Results. Observations were begun with a patient who has been shown to hemolyze transfused cells as well as her own at an

¹ Coombs, R. R. A., Mourant, A. E., and Race, R. R., *Brit. J. Exp. Path.*, 1945, **26**, 255.

² Boorman, K. E., Dodd, B. E., and Loutit, J. F., *Lancet*, 1946, **1**, 812.

³ Chown, B., *Am. J. Clin. Path.*, 1944, **14**, 114.

⁴ Landsteiner, K., and Miller, C. P., *J. Exp. Med.*, 1925, **42**, 853.

serum for the attachment of an incomplete antibody with negative results. Although these cells failed to show any evidence of sensitization after exposure to her serum *in vitro* at 37°C it was evident after repeated transfusions that these "compatible" cells shared in the rapid hemolysis *in vivo*. In other words, they were being destroyed by an immune body that could not be demonstrated in the patient's serum. It is probable that the immune body which caused the accelerated hemolysis in both patients was not demonstrable in the serum because it is adsorbed as it is produced by the large amount of cell antigen present.

It is not certain, however, that the particular body which was attached to the erythrocytes of both patients could not produce hemolysis or agglutination if present in sufficient concentration. Even if it is passive in this regard, the adherence of a protein to the cell surface probably exerts a detrimental effect by interfering with osmotic equilibrium. The spherocytosis observed may be produced by the accumulation of metabolites which raise the internal osmotic pressure and cause the absorption of fluid, thereby increasing osmotic and mechanical fragility of the erythrocyte and hastening its destruction. The failure to obtain agglutination of erythro-

cytes from patients with congenital hemolytic jaundice is compatible with the observations of Dacie and Mollison¹¹ that transfused cells have a normal longevity in this disease which suggests a cell defect rather than a hemolytic agent as the underlying cause.

Summary and Conclusions. The erythrocytes of 2 patients with acquired hemolytic anemia and spherocytosis have been shown to be readily agglutinated by an immune rabbit serum containing an antihuman globulin antibody. Since both patients have exhibited abnormal hemolytic activity for transfused cells this finding is compatible with the existence of an immune body type of hemolytic agent as the cause of the accelerated hemolysis.

Evidence has been presented to show that the immune substance can be separated from patients' cells by heating the cells to 56°C for 5 minutes and can then be combined with normal cells. The similarity of this immune body to the Rh blocking antibody is discussed. The demonstration of the presence or absence of an immune body attached to the erythrocytes should become a routine procedure in the study of hemolytic anemias.

¹¹ Dacie, J. V., and Mollison, P. L., *Lancet*, 1943, 1, 550.

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Role of Adrenalin in Recovery of Inhibited Conditioned Reactions.

ERNST GELLHORN.*

From the Laboratory of Neurophysiology, Department of Physiology, University of Minnesota, Minneapolis.

It was shown in earlier papers from this laboratory^{1,2} that insulin coma and chemical- or electrically-induced convulsions cause the reappearance of previously inhibited con-

ditioned reactions (c.r.). No distinct effect was exerted on positive c.r.'s by these procedures.^{3,4} Since it had been shown previously^{5,6} that the action which these proce-

* Aided by a grant from the Josiah Macy, Jr., Foundation.

¹ Gellhorn, E., and Minatoya, H. J., *J. Neurophysiol.*, 1943, 6, 161.

² Kessler, M., and Gellhorn, E., *Am. J. Psychiat.*, 1943, 99, 687.

³ Gellhorn, E., *Proc. Soc. Exp. Biol. and Med.*, 1945, 59, 155.

⁴ Gellhorn, E., *Arch. Neurol. and Psychiat.*, 1946, 50, 216.

⁵ Gellhorn, E., *Arch. Neurol. and Psychiat.*, 1938, 40, 225.

TABLE II.

Demonstration of an Immune Body in a Saline Eluate from the Cells of a Second Patient with Acquired Hemolytic Anemia. After 18 hr at 5°C the cells showed more intense agglutination in the anti-globulin serum after an additional incubation at 37°C.

	Group O cells 0.1 cc	Anti-globulin rabbit serum	
		18 hr ice box	+ 2 hr 37°C
Eluate from patient's cells 1.0 cc	1	±	++
	2	±	++
	3	±	++
Eluate from normal cells 1.0 cc	1	0	0
	2	0	0
	3	0	0

different from that of cells of normal individuals in being readily agglutinated by the antiglobulin rabbit serum. This susceptibility to agglutination has been observed so far only in cells that have been exposed to the Rh "blocking" antibody and presumably coated with an immune globulin which attaches to the cell but does not produce agglutination in saline suspension. This suggests that there is an immune body present in acquired hemolytic anemias which attaches to the cell but does not produce agglutination under conditions in which the cells are usually observed. This analogy was further borne out when it was observed that the washed erythrocytes from these patients became agglutinated when placed in 30% beef albumin and 2.0% acacia as are cells known to be sensitized with the Rh "blocking" antibody.^{6,7} However, the patient's cells did not agglutinate when suspended in human serum as do cells sensitized by the Rh "blocking" antibody.⁸

The failure to remove the factor responsible for the agglutination of the cells by repeated washings and the ready removal by heating to 56°C again suggests a cell antibody combination.⁴ In addition, the demonstration that a substance was released at 56°C which was then capable of attaching to normal cells and withstanding repeated washing further identifies the substance as an erythrocyte antibody. The transfer of

the Rh blocking antibody has been carried out by this method.^{9,10}

Only tentative explanation can be offered for the temperature effects which seemed to be important in the transfer of the antibodies to normal cells. It is possible that adsorption of the immune globulin on the cells proceeded better in the cold and that subsequent incubation at higher temperatures was necessary for fixation so as to prevent removal by repeated washing.

Although an antibody could be detected on the surface of the erythrocyte from the 2 patients it could not be demonstrated in the serum. The first patient has never shown evidence of an atypical agglutinin or hemolysin in the serum, and moreover group O cells which were suspended in her serum at 37°C failed to agglutinate when washed and suspended in the antiglobulin serum. The second patient exhibited a relatively low titer of cold agglutinins and an atypical agglutinin of an undetermined specificity active at 37°C. The origin of this atypical agglutinin is not clear since the patient had not had transfusions prior to the onset of her illness and a single pregnancy had terminated at the end of 4 months. While the specificity of this agglutinin remained to be determined there is evidence that it was not responsible for the hemolytic process. The group O cells which were not agglutinated by the patient's serum and judged to be compatible were washed and tested with the antiglobulin

⁶ Diamond, L. K., and Denton, R. L., *J. Lab. and Clin. Med.*, 1945, **30**, 821.

⁷ Levine, P., *Am. J. Clin. Path.*, 1946, **16**, 597.

⁸ Wiener, A. S., *J. Lab. and Clin. Med.*, 1945, **30**, 662.

⁹ Haberman, S., and Hill, J. M., *Texas State J. Med.*, 1944, **40**, 182.

¹⁰ Carter, B. B., and Loughrey, J., *Am. J. Clin. Path.*, 1945, **15**, 575.

citability of these centers to normal (homeostasis, Darrow and Gellhorn).¹³

The statement by Himwich and Fazekas¹⁴ that my theory of the shock treatment in schizophrenia does not hold since adrenalin is ineffective in the treatment of schizophrenics[†] is obviously based on a gross misunderstanding. The core of the theory (cf. also Gellhorn^{4,6}) is not that insulin coma and electrically- or chemically induced convulsions act through the secretion of adrenalin but that they greatly increase the reactivity of autonomic centers which in turn may influence the brain in such a manner as to restore normal behavior.[‡] The neu-

rological basis for such a hypothalamic-cortical relationship has been elucidated by Murphy and Gellhorn whereas the changes in conditioned reflexes described in this series of papers indicate at least one of the mechanisms which contribute to an altered behavior.

Summary. Conditioned reactions which had been inhibited by lack of reinforcement in rats may be restored by insulin coma in normal as well as in adrenomedullated rats. Apparently a secretion of adrenalin which is observed under conditions of insulin coma and electroshock does not play an integral part in the mechanism of the restoration of inhibited conditioned reactions. This interpretation is supported by the fact that the injection of adrenalin remains ineffective in the normal animals as far as restoration of inhibited conditioned reactions is concerned, whereas insulin coma and electroshock restore these reactions. It is inferred from these experiments that the restitution of inhibited conditioned reactions is linked up with a hypothalamic-cortical discharge previously established by Murphy and Gellhorn and is not due to the liberation of adrenalin which invariably accompanies the excitation of hypothalamic centers under conditions of insulin coma and electroshock.

¹³ Darrow, C. W., and Gellhorn, E., *Am. J. Physiol.*, 1939, **128**, 185.

¹⁴ Himwich, H. E., and Fazekas, J. F., *Arch. Neurol. and Psychiat.*, 1942, **47**, 800.

[†] The paper by Dynes and Tod¹⁵ to which they refer does not give any evidence for or against the adrenalin treatment of schizophrenics but points out that adrenalin injections cause less emotional reactions (anxiety and fear) in the schizophrenic than in the normal individual.

¹⁵ Dynes, J. D., and Tod, H., *J. Neurol. and Psychiat.*, 1940, **3**, 1.

[‡] The secretion of adrenalin is but one symptom of this central effect and probably of minor importance.

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Biological Value of Proteins Determined With *Tetrahymena geleii* H.*

MAX S. DUNN AND LOUIS B. ROCKLAND.

From the Chemical Laboratory, University of California, Los Angeles.

The possibility that the ciliated protozoan, *Tetrahymena geleii* H., might be used to determine the biological value of proteins was

* Paper 40. For Paper 39, see Rockland and Dunn.¹ This work was aided by grants from the National Institute of Health and the University of California.

The biological value of proteins has been determined with chickens, dogs, rabbits, rats, swine, and humans by the classical McCollum, Osborne and Mitchell methods as well as by newer techniques

suggested by the observations that this small animal utilizes unhydrolyzed protein¹² and that its amino acid requirements¹³ approx-

described by Caunon *et al.*² and by Harrison and Long.³ Reviews have been given by Mitchell,⁴ Madden and Whipple,⁵ Barnes *et al.*,⁶ Cahill,⁷ Boas-Fixen,^{8,9} Allison *et al.*,¹⁰ and Bloek and Mitchell.¹¹

¹ Rockland, L. B., and Dunn, M. S., in press (No. 39).

² Cannon, P. P., Humphreys, E. M., Wissler, R. W., and Frazier, L. E., *J. Clin. Invest.*, 1944,

dures have in common is the excitation of the centers of the sympatheticoadrenal system, it was thought that the restoration of inhibited c.r.'s was likewise due to this increased reactivity of sympathetic centers. In order to clarify further the mechanism involved an attempt was made to determine whether the hypothalamic-cortical discharge, which occurs on excitation of diencephalic autonomic centers, (Murphy and Gellhorn)⁷ or the release of adrenalin was responsible for the reestablishment of inhibited c.r.'s. For this purpose 2 series of experiments were performed. In the first, adrenalin was injected into normal animals whereas in the second group insulin coma was produced in adrenalectomized animals. In both groups the effect of these procedures to restore c.r.'s which had been inhibited by lack of reinforcement was studied.

Methods. All experiments were performed on rats in which a definite escape reaction, as used in our earlier studies, had been established in response to the conditioned stimulus (bell or light). After the c.r. had been established it was inhibited by lack of reinforcement. Adrenalin (.2 mg per kg) was injected intraperitoneally 3 or 4 times on alternate days. The effect of these injections was compared with that induced by insulin coma or electroshock on 6 normal animals. The action of insulin coma was likewise studied in 6 adrenalectomized rats. For further detail compare the literature cited above and the paper by Arnett and Gellhorn.⁸

Results and Discussion. Nineteen injections of adrenalin were performed on 6 normal rats in which preceding or following the adrenalin series the response of the rat to insulin coma or electroshock was studied. In all but one experiment no effect was elicited on the inhibited c.r.'s by the injection of adrenalin although the animals showed the

typical recovery reaction under the influence of insulin coma or electroshock. In the one case in which the response of the c.r.'s was increased to 50% by adrenalin the effect was restricted to one day, whereas a single injection of insulin leading to convulsion elicited in the same animal a marked recovery of the c.r.'s which lasted for 4 days and reached 90%. The experiments performed on adrenalectomized rats led to a recovery (50% to 90%) of the c.r.'s after induction of an insulin coma. These experiments prove conclusively the fundamental difference between the effects of injected adrenalin and those of insulin coma and electroshock. Since in the latter conditions adrenalin is likewise involved, being secreted as the result of excitation of sympathetic centers, the difference, as far as restitution of inhibited c.r.'s is concerned, is based on a central action which occurs in insulin coma and electroshock and is not related to adrenalin.

The answer to this problem seems to lie in altered hypothalamic-cortical relations. Obrador,⁹ Kennard,¹⁰ and Murphy and Gellhorn⁷ have demonstrated the profound effect which stimulation and destruction of the hypothalamus exert on electrical cortical activity. It may be assumed on this basis and on clinical evidence concerning autonomic relations in psychotic patients⁶ that quantitative alterations in the activity of autonomic centers will in turn influence cortical functions.

It may be said that the reactivity of autonomic centers is greatly increased in insulin hypoglycemia¹¹ and in experimental convulsions.¹² This must lead to quantitative changes in cortical activity which may be the basis of alterations in behavior. Adrenalin, however, whether injected or secreted, far from causing a similar reaction, seems to depress autonomic centers and may be looked upon as a means of restoring increased ex-

⁶ Gellhorn, E., *Autonomic Regulations*, New York, 1943.

⁷ Murphy, J. P., and Gellhorn, E., *J. Neurophysiol.*, 1945, **8**, 341 and 431.

⁸ Arnett, V., Kessler, M., and Gellhorn, E., *Am. J. Physiol.*, 1942, **137**, 653.

⁹ Obrador, S., *J. Neurophysiol.*, 1942, **6**, 81.

¹⁰ Kennard, M. A., *J. Neurophysiol.*, 1943, **6**, 405.

¹¹ Gellhorn, E., Ingraham, R. C., and Moldovsky, L., *J. Neurophysiol.*, 1938, **1**, 301.

¹² Gellhorn, E., and Darrow, C. W., *Arch. Internat. Pharmacodyn.*, 1939, **62**, 114.

TABLE I.
Biological Values of Three Proteins.

Criterion	Animal	Protein			Reference No.
		Casein	Lactalbumin	Gelatin	
Liver wt regeneration	Rat	100	100	0	3
Nitrogen balance	Dog	100	137		14
Plasma protein regeneration	"	100	83		15
Protein level for optimum wt increase	Rat	100	133		16
Wt increase	Chick	100		46	17
" "	Rat	100	100-206*		16
Acid production	<i>T. geleii</i> H	100	61-112*	0-2*	This paper

* Range of values at different levels of sample.

washed suspension of *Tetrahymena geleii* H cells, and incubated at about 25° (room temperature) for varying lengths of time. The response of the organism was measured by electrometric titration with standard base.

Discussion. The biological values were calculated from the titration data using casein as the standard. The values found for lactalbumin, a protein hydrolyzate and gelatin varied with the incubation time and, as shown in Fig. 1, with the level of nitrogen in the sample. It has been shown previously by McCollum *et al.*¹⁵ and Plimmer *et al.*¹⁹ that biological values vary with the time and by Osborne *et al.*,¹⁶ Barnes *et al.*⁶ and other workers cited by these authors that they vary with the level of the protein.

¹⁴ Melnick, D., and Cowgill, G. R., *J. Nutrit.*, 1937, **13**, 401.

¹⁵ Melnick, D., Cowgill, G. R., and Burack, E., *J. Exp. Med.*, 1936, **64**, 897.

¹⁶ Osborne, T. B., Mendel, L. B., and Ferry, E. L., *J. Biol. Chem.*, 1919, **37**, 223.

¹⁷ Almquist, H. J., Stokstad, E. L. R., and Halbrook, E. R., *J. Nutrit.*, 1935, **10**, 193.

¹⁸ McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, **23**, 231.

¹⁹ Plimmer, R. H. A., Rosedale, J. L., Raymond, W. H., and Lowndes, J., *Biochem. J.*, 1934, **28**, 1963.

The present data for 3 proteins, as well as the values reported in the literature, are summarized in Table I. The biological values found at some levels of samples by the authors' micro method are in approximate agreement with those obtained by other procedures. Although this correlation is of interest, it appears more significant that the true biological value of a protein is best expressed by a series of numbers if time, level of sample and possibly other variables are to be taken into account. It may be suggested that biological values of most meaning may be represented by areas under curves or volumes under planes. Whether or not there is a direct, or any, relation between the human requirements for proteins and the biological values of proteins determined with protozoa or other animals remains to be determined.

Further studies of this problem are in progress.

Summary. A method for the determination of biological values of proteins with *Tetrahymena geleii* H has been described. The values found for casein, lactalbumin, a commercial protein hydrolyzate and gelatin have been compared with each other and with those reported in the literature.

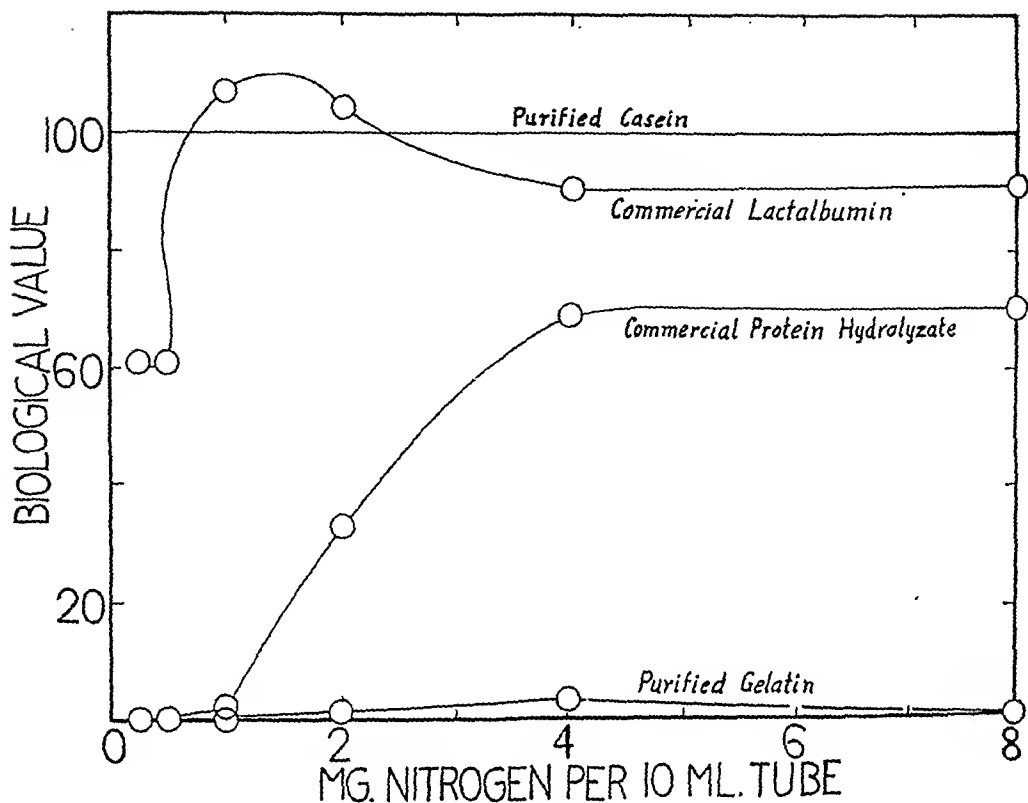


FIG. 1.

Curves relating biological value and nitrogen of proteins.

imate those of some mammals. It seems evident that biological values could be deter-

23, 601.

³ Harrison, H. C., and Long, C. N. H., *J. Biol. Chem.*, 1945, **161**, 545.

⁴ Mitchell, H. H., *Physiol. Rev.*, 1924, **4**, 424.

⁵ Madden, S. C., and Whipple, G. H., *Physiol. Rev.*, 1940, **20**, 194.

⁶ Barnes, R. H., Bates, M. J., and Maack, J. E., *J. Nutrit.*, 1946, **32**, 535.

⁷ Cahill, W. M., *J. Am. Diet. Assn.*, 1945, **21**, 433.

⁸ Boas-Fixen, M. A., *Biochem. J.*, 1934, **28**, 592.

⁹ Boas-Fixen, M. A., *Nutr. Abstr. Rev.*, 1934-35, **4**, 447.

¹⁰ Allison, J. B., and Collaborators, *Annals New York Acad. Sci.*, 1946, **47**, 241.

¹¹ Block, R. J., and Mitchell, H. H., *Nutr. Abstr. Rev.*, 1946-1947, **16**, 249.

¹² Rockland, L. B., and Dunn, M. S., *Arch. Biochem.*, 1946, **11**, 541.

¹³ Kidder, G. W., and Dewey, V. C., *Physiol. Zool.*, 1945, **15**, 136.

mined with greater speed, convenience and economy with this organism than with larger animals and that errors due to biological variation would be minimized because of the large number of individuals and the aseptic conditions employed.

Experimental. A protein-free basal medium similar to that described by Kidder and Dewey,¹³ except for the omission of the amino acids, was employed in the present experiments. The technics were similar to those used in the authors' laboratory for the determination of amino acids with lactic acid bacteria. Aliquots of protein solutions, homogenized suspensions or hydrolyzates were added to 19 x 100 mm pyrex test tubes. Ten levels of sample, each in 2 or more tubes, were used. Protein- and amino acid-free medium and distilled water were added to a total volume of 10 ml per tube. The tubes were plugged, sterilized, inoculated with a

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¹⁷ Almquist, H. J., Stokstad, E. L. R., and Halbrook, E. R., *J. Nutrit.*, 1935, **10**, 193.

¹⁸ McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, **23**, 231.

¹⁹ Plimmer, R. H. A., Rosedale, J. L., Raymond, W. H., and Lowndes, J., *Biochem. J.*, 1934, **28**, 1963.

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Treatment of Paroxysmal Auricular or Nodal Tachycardia With the Vasopressor Drug, Neosynephrine.

W. B. YOUMANS, MORTON J. GOODMAN, AND JARVIS GOULD.

From the Departments of Physiology and Medicine, University of Oregon Medical School, Portland, Oregon.

The cardio-inhibitory reflexes elicited by a rise in blood pressure are strikingly sensitive in unanesthetized animals.¹ Therefore, it was considered that a moderate, sudden, brief rise in blood pressure produced by intravenous injection of a vasoconstrictor drug might reflexly stop a paroxysmal auricular or nodal tachycardia.

Neosynephrine (laevo- α -hydroxy- β -methyl-amino-3-hydroxy-ethylbenzine hydrochloride) has a relatively high pressor potency as compared with its direct cardiac stimulating potency.^{2,3} Following intravenous injection in normal human subjects, an amount which produces a moderate increase in blood pressure causes a striking bradycardia.^{4,5} Therefore, neosynephrine was chosen as a favorable vasopressor compound to be given a trial in the treatment of paroxysmal tachycardia. Neosynephrine has been given to several patients having cardiac arrhythmias, including 2 with paroxysmal auricular tachycardia, and these 2 reverted to normal sinus rhythm.⁴

Results. Neosynephrine was given intravenously to each of 4 patients with idiopathic paroxysmal tachycardia of supraventricular origin. Each had a normal blood pressure between attacks and a somewhat lower blood pressure during attacks. Electrocardiographic records were taken and blood pressure was recorded repeatedly during and following the injections with the patient in the supine posi-

tion. In each case the neosynephrine injection was completed within 30 seconds. The first patient had tachycardia which had persisted for several hours in spite of attempts to stop it with carotid sinus pressure, ocular pressure, amytal, and mecholyl. Her blood pressure was 100/70 during the attack. When she was given neosynephrine, the systolic pressure increased to 140 mm Hg within 60 seconds and the rhythm reverted to normal. The second patient had an attack that had persisted for 20 hours in spite of the usual mechanical methods of treatment and the administration of mecholyl. The patient was given 4 doses of neosynephrine intravenously, 0.15 mg, 0.30 mg, 0.50 mg and 0.80 mg respectively, with ample time for the blood pressure to return to the preinjection level after each dose. The first 3 doses were ineffective. When the dose of 0.8 mg was given, the blood pressure rose from 110 to 160 mm of Hg and the rhythm reverted to normal 45 seconds after the beginning of the injection. The third patient had had the tachycardia for several hours before she was seen. She was given 0.4 mg of neosynephrine; the systolic blood pressure rose from 110 to 140 mm of Hg and the rhythm reverted to normal within the first minute after the injection. The fourth patient was a man who had had frequent attacks which did not stop spontaneously but could be stopped by the Valsalva maneuver. A dose of 0.5 mg of neosynephrine failed to restore the normal rhythm. A dose of 0.80 mg increased his systolic blood pressure from 90 to 130 Hg in 50 seconds at which time the rhythm reverted to normal.

Over-dosage with neosynephrine must be avoided because of the danger of producing an excessively high blood pressure. Occasional ventricular beats were seen with the

¹ Haney, H. F., Lindgren, A. J., Karstens, A. I., and Youmans, W. B., *Am. J. Physiol.*, 1943, **130**, 675.

² Youmans, W. B., Haney, H. F., and Aumann, K. W., *Am. J. Physiol.*, 1940, **130**, 190.

³ Keys, A., and Violante, A., *Proc. Soc. Exp. Biol. and Med.*, 1940, **44**, 4.

⁴ Keys, A., and Violante, A., *J. Clin. Invest.*, 1942, **21**, 1.

⁵ Youmans, W. B., Gould, Jarvis, and Goodman, Morton J., experiments in progress.

larger doses used. However, experiments with animals have demonstrated that neosynephrine does not produce ventricular fibrillation even in relatively high doses in animals sensitized by cyclopropane anesthesia.⁶ If a given dose is ineffective, a larger dose may be given after a 10-minute interval until the rhythm is reverted or until a rise of systolic pressure up to about 160 mm of Hg is produced. Doses between 0.5 mg and 1.0 mg should be effective in most cases.

Two of the 4 patients complained of mild precordial discomfort for a few minutes following the injection. This was the only unpleasant effect noted. Each patient described either a sensation of coolness of the skin or a tingling sensation evidently produced by the cutaneous vasoconstriction and piloerection.

Summary. Four nonhypertensive patients

⁶Meek, W. J., *Harvey Lect.*, Ser. XXXVI, 1940-41, 188.

with idiopathic paroxysmal tachycardia of supraventricular origin were given neosynephrine intravenously in amounts sufficient to increase the systolic blood pressure 30 to 40 mm of Hg. In 2 of these the attacks had persisted for several hours in spite of all mechanical methods of treatment and administration of mecholyl. In all 4 cases the rhythm reverted to normal within one minute after the injection of neosynephrine. This is considered to be produced by reflexes from the carotid sinuses and aortic arch. Occasional ventricular beats occurred during the 2-minute period after the return to the normal sinus rhythm. Blood pressure returned to normal within 4 to 8 minutes after the injection.

Intravenous injection of neosynephrine or some other brief-acting vasopressor compound may prove to be the treatment of choice in selected cases of auricular or nodal paroxysmal tachycardia.

15802

Dark-field Observations on Lymphocytes Exposed to X-Rays and Other Injurious Agents.*

ROBERT SCHREK.

From the Tumor Research Unit, Veterans Administration Hospital, Hines, Ill.

By the method of unstained cell counts, it has been shown^{1,2} that X-rays had a delayed cytotoxic action on lymphocytes irradiated *in vitro* and incubated at 37°C. The death of the cells was preceded by irregularities in the shape and the refractivity of the cells. In the present study, these morphologic changes were studied by dark-field illumination with a cardioid condenser.

* Published with the permission of the Chief Medical Director, Department of Medicine and Surgery, Veterans Administration, who assumes no responsibility for the opinions expressed or conclusions drawn by the author.

¹Schrek, R., *Radiology*, 1946, 46, 395.

²Schrek, R., *J. Cell. and Comp. Physiol.*, 1946, 28, 277.

On dark-field examination of a fresh suspension of the thymus of a rabbit, most cells appeared filled with fine intranuclear granules and a few cytoplasmic mitochondria (Fig. 1). After irradiation of the suspension and incubation for 3.5 hours, about 32% of the cells developed one or a few small, dark, round structures which may be termed primary vacuoles (Fig. 2). On dark-field examination, it was not possible to determine whether these structures were in the nucleus or cytoplasm. The vacuoles occasionally increased in size during observation. Some of them were surrounded by thick, bright, circular walls. They caused the distortion of the shape of many cells. They were present in viable cells which resisted staining with

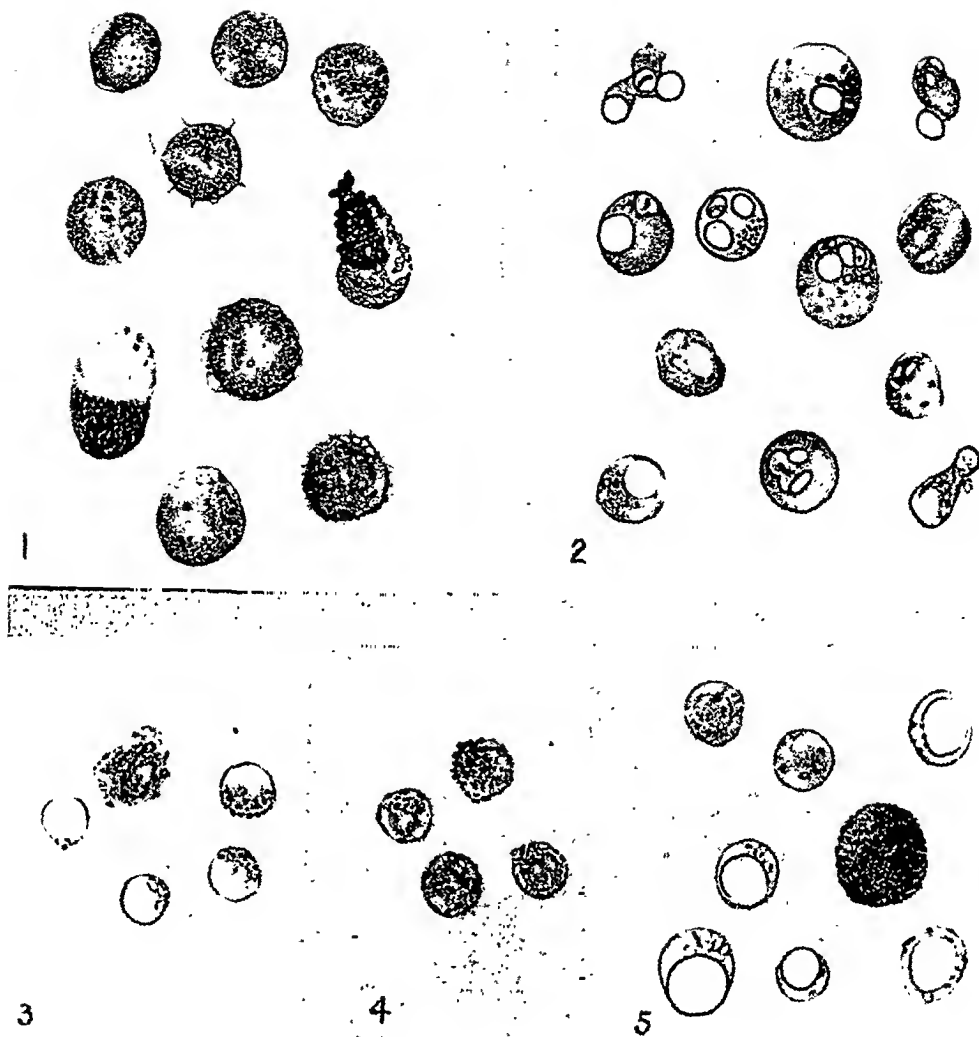


FIG. 1. Cells in a suspension derived from the thymus of a rabbit. The cells have innumerable fine intranuclear granules and a few larger mitochondria. A few cells show degenerative changes—small cytoplasmic bullae and thin cytoplasmic projections. Two granulocytes are shown.

FIG. 2. Thymic cell suspension, radiated (1000 r) and incubated for 3 hours at 37°C. The cells have one or more primary vacuoles, some of which are surrounded by bright vacuolar walls. A few cells are distorted.

FIG. 3. Thymic cell suspension, radiated (1000 r) and incubated 24 hours at 37°C. The cells are small, have relatively large secondary vacuoles and crescents of small and large granules. The cell walls are distinct. One normal granulocyte is present.

FIG. 4. Thymic cell suspension incubated for 3 hours at 45°C. The cells are small with fine and coarse granules.

FIG. 5. Thymic cell suspension treated with an equal volume of distilled water. The nuclei are dark, free of granules and have a distinct nuclear wall. The granulocyte appears normal. Magnification in all figures is 1250 X.

eosin. The spherical vacuolated cells had a greater average diameter than the non-vacuolated.

In histologic sections of the suspension, approximately 12% of the nuclei had large, acidophilic or slightly basophilic, Feulgen-

TABLE I.
A Comparison of the Characteristics of Primary and Secondary Vacuoles.

Method of study	Primary or early vacuoles	Secondary or late vacuoles
Dark-field examination		
Cell	Round or irregular Usually increased in size Usually no cell wall	Round Usually small Cell wall present
Vacuole	Single or multiple Small or large Usually have vacuolar wall	Single Relatively large No vacuolar wall
Histologic section	Intranuclear acidophilic area	Pyknotic nucleus
Method of unstained cell count	Usually in viable cells	In dead cells

negative vacuoles surrounded by dense chromatin material in the shape of a ring, horse-shoe or crescent. The intranuclear vacuoles in histologic sections correspond to the primary vacuoles observed on dark-field examination.

Similar vacuoles were observed both on dark-field examination and in histologic sections of rat thymic tissue excised 3 hours after irradiation *in vivo*.

On dark-field examination of a suspension irradiated *in vitro* and incubated 24 hours, 86% of the cells were small, had a thick, bright cell wall and a crescentic, polar mass of fine and coarse granules. Each of the cells also had a single, relatively large, round or oval, dark structure which was termed a secondary vacuole (Fig. 3). Cells with secondary vacuoles stained readily with eosin and were evidently dead. In stained sections, most cells had pyknotic nuclei which corresponded with the secondary vacuoles seen on dark-field examination.

A comparison of the primary and secondary vacuoles is given in Table I. It was usually possible to differentiate the 2 types of vacuoles on dark-field examination but intermediate forms were sometimes observed.

Nonirradiated suspensions incubated at 37°C also developed cells with primary and secondary vacuoles, but the rate of formation of the vacuolated cells was much slower. Apparently, irradiated and nonirradiated cells incubated at 37°C were killed by the same mechanism but irradiation accelerated the normal degenerative process.

In suspensions incubated at 45°C for 3 hours, the cells were dead and were filled

with granules which appeared coarser and brighter than in the unincubated cells (Fig. 4). On histologic examination, many nuclei had large chromatin masses. The cells incubated at 45°C were apparently killed by a different mechanism (coagulation of protein?) than those incubated at 37°C. Furthermore, cytologic changes produced by heat were different from those induced by X-rays.

Irradiated cells incubated at 45°C appeared morphologically identical and survived as long as nonirradiated ones. Radiation did not accelerate the cellular degenerative processes at 45°C.

In view of Failla's³ theory that X-rays cause the absorption of water into the nucleus, it is of interest to compare the effects of distilled water and of X-rays on lymphocytes. Addition of water to an equal volume of thymic cell suspension caused an immediate enlargement of the cells and a change in the nuclei which lost their granular appearance and became dark ill-defined areas (Fig. 5). Later the nuclear walls became visible as thin, bright lines. Histologic sections of thymic tissue incubated with water showed cells with vacuolated nuclei (Fig. 1 in Schrek⁴). In contrast, irradiated and incubated (37°C) cells in an isotonic solution suffered no morphologic change for 2 hours or more. After the latent period, there appeared small, dark, intranuclear vacuoles which enlarged and developed bright, vacu-

³ Failla, G., in Ward, H. B., *Some Fundamental Aspects of the Cancer Problem*, The Science Press, New York, 1937, p. 202.

⁴ Schrek, R., *Am. J. Path.*, 1945, **21**, 1101.

olar walls. Histologic sections showed vacuolated nuclei.

From the reports in the literature and the findings in this laboratory, it is possible to formulate a hypothesis on the action of X-rays on lymphocytes. Presumably, the radiation produced, in one or more foci in the nucleus, first a photochemical change, then a chemical reaction which caused the absorption of water into these foci with the formation of primary vacuoles. The vacuoles enlarged forcing the chromatin material peripherally. The ring of chromatin ruptured at one point and then contracted re-

sulting in a horseshoe shaped mass, a crescent and ultimately a small, round pyknotic nucleus.

Summary. Incubation of lymphocytes in suspension at 37°C produced primary and secondary vacuoles visible on dark-field examination. Preliminary irradiation of the suspensions greatly accelerated this process. The addition of distilled water to lymphocytes produced enlargement and vacuolization of the nuclei. The hypothesis is proposed that irradiation caused the absorption of water into one or more foci in the nucleus.

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SECTION MEETINGS

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February 14, 1947

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Northwestern University

March 4, 1947

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University of Southern California

March 14, 1947

15803

Human Infection with Rift Valley Fever Virus and Immunity Twelve
Years After Single Attack.*

ALBERT B. SABIN AND RICHARD W. BLUMBERG.

From the Children's Hospital Research Foundation and Department of Pediatrics, University of Cincinnati College of Medicine.

The virus of Rift Valley Fever (a disease of sheep and cattle in Kenya Colony, British East Africa) was first isolated in 1930 by Daubney, Hudson and Garnham,¹ who also established its pathogenicity for human beings. Since that time there have been accidental human infections in practically every laboratory in which this virus has been studied—4 cases in Kenya¹ in 1930, 4 cases in 1931 in London² and another in 1934,³

in New York 1 case in 1932,⁴ 3 cases in 1933⁵ and 3 cases in 1934,⁶ and at least 1 case in Uganda⁷ in 1944—a total of 17. The purpose of the present communication is not merely to record still another instance of accidental laboratory infection, but rather to indicate (a) that a strain of virus which has had at least about 300 brain to brain passages in mice has not undergone any modification in the type of disease it can produce in human beings, and (b) that neutralizing antibodies for the virus can persist for at least 12 years after a single attack of

* This investigation was conducted with the aid of the Commission on Virus and Rickettsial Diseases, Army Epidemiological Board, Office of the Surgeon General, U. S. Army, Washington, D.C.

¹ Daubney, R., Hudson, J. R., and Garnham, P. C., *J. Path. and Bact.*, 1931, **34**, 545.

² Findlay, G. M., *Tr. Roy. Soc. Trop. Med. and Hyg.*, 1932, **25**, 229.

³ Findlay, G. M., quoted by Kitchen.⁵

⁴ Schwentker, F. F., and Rivers, T. M., *J. Exp. Med.*, 1934, **59**, 305.

⁵ Kitchen, S. F., *Am. J. Trop. Med.*, 1934, **14**, 547.

⁶ Francis, T., Jr., and Magill, T. P., *J. Exp. Med.*, 1935, **62**, 433.

⁷ Smithburn, K. C., personal communication concerning case at Yellow Fever Research Institute, Entebbe, Uganda, East Africa.

ACCIDENTAL HUMAN LABORATORY INFECTION WITH RIFT VALLEY FEVER VIRUS
WHICH HAS UNDERGONE ABOUT 300 OR MORE INTRACEREBRAL PASSAGES IN MICE

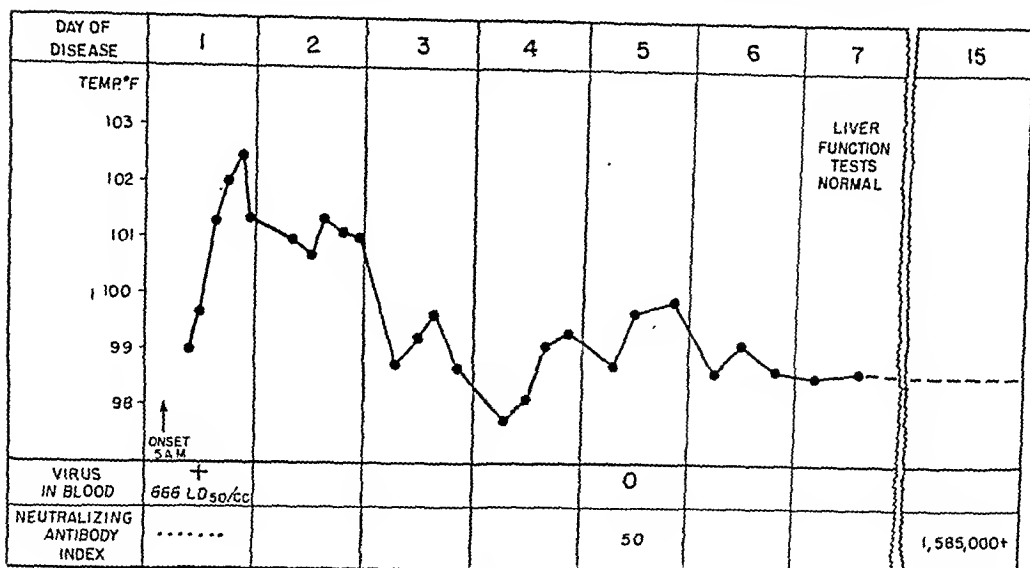


FIG. 1.

the disease without further exposure to the virus.

The "K" strain of virus was obtained from Dr. N. Ishii of Japan in 1945 and was alleged to be a strain of mouse-adapted dengue virus. However, studies, which will be reported at a later date, established that the "K" virus was a strain of Rift Valley Fever virus. The history of the virus prior to the time it came to be called "dengue" is not known, although it is known that Rift Valley Fever virus was brought into Japan from France for laboratory investigations. It was stated that the "K" virus had undergone 293 brain to brain passages in mice since 1942 and it had had 5 additional intracerebral passages in mice in Cincinnati, when the accidental human infection to be reported here had occurred. One of us (A.B.S.) worked continuously with this virus from October 26, 1945 until early January, 1946, without becoming infected or developing neutralizing antibodies for the virus. He resumed work with this virus on October 31, 1946 when he ground a number of infected mouse brains in a mortar and performed an intra-abdominal neutralization test in mice, at which time contamination of the skin or aspiration of

droplets of virus might have occurred. Between October 31 and the onset of illness on November 6, the only other activity related to this virus consisted of the daily examination of the inoculated mice.

A.B.S., who had been in good health, was awakened from his sleep at 5 a. m. on November 6 by a very severe frontal headache. Acetylsalicylic acid and codeine were taken at that time and since they brought no relief, no more was taken during the course of the illness so that the temperature curve (rectal temperatures shown in Fig. 1) was not subsequently affected by antipyretics. The temperature did not rise until the late afternoon and evening. Severe aching in the muscles, bones, and joints, and anorexia were the only other symptoms which developed during the course of the day and persisted for about 48 hours. The temperature returned to normal on the 3rd day and only mild muscle aching and weakness remained. He was out of bed on the 4th day and back in the laboratory on the 5th day. The low-grade fever up to 99.9° on the 5th day was associated with a feeling of feverishness and slight malaise, but recovery was complete and uneventful thereafter. It may be of interest

TABLE I.
Immunological Identity of Virus Recovered from Patient "A.B.S." and "K" Strain of Rift Valley Fever Virus.

Dilution of virus in mixture	Intra-abdominal Neutralization Test in Mice. Mortality in mixture with:	
	Normal rabbit serum	"K" hyperimmune rabbit serum
10-1	—	0, 0, 0, 0
10-2	1+, 1+, 1+, 1+	0, 0, 0, 0
10-3	1+, 1+, 1+, 1+	0, 0, 0, 0
10-4	2, 2, 2, 3	0, 0, 0, 0
10-5	2, 2, 2, 2	0, 0, 0, 0
10-6	2, 2, 0, 0	0, 0, 0, 0
10-7	3, 0, 0, 0	0, 0, 0, 0

* Each digit represents day of death of each mouse used in the test; 1+ = more than 24 but less than 36 hours; 0 = survival.

to note that the following tests, for which we are indebted to Dr. S. Rapoport, carried out on the blood 6 days after onset revealed no abnormality in liver function: Total bilirubin—0.3 mg %; cephalin flocculation negative; phosphorus—3.2 mg % and phosphatase—3.2 Bodansky units; total protein—7.6 g % with an albumin-globulin ratio of 2.8.

Approximately 10 hours after the first appearance of headache, blood was drawn and inoculated in mice—0.5 cc of defibrinated blood intra-abdominally into each of 5 mice and 0.03 cc of the serum intracerebrally into each of 5 additional mice. All of these mice succumbed within 48 hours, and 3 successful serial passages by the intra-abdominal or intracerebral routes were carried out. Extensive hepatic necrosis and acidophilic intranuclear inclusions were present in the liver cells of the first passage mice. The immunological identity of the virus recovered from the blood of A.B.S. and the "K" strain of Rift Valley Fever virus was established by the results of the intra-abdominal neutralization test shown in Table I. Varying dilutions of mouse brain suspension containing the 3rd passage of the "A.B.S." virus were mixed with equal parts of "K" hyperimmune rabbit serum and for control a similar set was prepared with normal rabbit serum. After incubation of the mixtures in a water bath at 37°C for about one hour, 0.1 cc was injected intra-abdominally into approximately 4-week-old Swiss mice. Some of this

TABLE II.
Early Development and Long Persistence of Neutralizing Antibodies for Rift Valley Fever Virus ("K" Strain) After a Single Attack of the Disease in Human Beings.

Neutralization Tests by Intra-abdominal Route in Mice.											
Test	Serum	Interval between onset and specimen	Mortality at indicated dilutions of virus in mixtures							Neutralization index	
			10-1	10-2	10-3	10-4	10-5	10-6	10-7		
A	Normal rabbit control	—	—	—	—	2,2,3,3,1	2,2,2,2	2,2,2,5	6,0,0,0	10-6.7	—
	Patient A.B.S.—1946	4 days	2,2,2,2	2,2,2,2	2,2,2,2	2,2,2,2	2,3,0,0	0,0,0,0	—	10-5.0	50
	Patient A.B.S.—1946	14 "	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	—	10-6.5	? 1,585,000+?
	R.W.B.—1946	Specimen 1	2,2,2,2	2,2,2,2	2,2,2,2	2,2,2,2	2,2,2,2	2,3,3,5	—	10-6.5	? 5
B	Normal rabbit control	Specimen 2, 13 days later	2,2,2,2	2,2,2,2	2,2,2,2	2,2,2,2	2,2,2,2	2,2,2,0	0,0,0,0	10-5.8	? 8
	Normal rabbit control	—	—	—	2,2,2,2	2,2,2,2	2,2,2,2	2,2,2,2	0,0,0,0	10-6.5	—
	Patient K.C.S.—1944	16 mo	—	—	5,8*,0,0	7,0,0,0	0,0,0,0	0,0,0,0	—	10-3.5	1,000
	Patient T.F.—1934	12 yrs	6,8,4,10,0	4,6,6,0	8*,10,0,0	7,10,0,0	0,0,0,0	0,0,0,0	—	10-3.0	3,160

+ Each digit represents day of death of each mouse used in the tests. 0 = survival.

* Rift Valley Fever virus shown to be cause of death by passage of liver.

† No virus recovered by passage of liver.

same preparation of "K" hyperimmune rabbit serum was sent to Dr. Kenneth Smithburn at the Yellow Fever Research Institute in East Africa who reported that it neutralized their viscerotropic Rift Valley Fever virus.

In order to determine how much virus was present in the blood at the beginning of the human infection, some of the serum obtained 10 hours after onset and kept frozen in a CO₂ box was titrated by intracerebral inoculation of mice. It was rather surprising to find only 666 LD₅₀ of virus per cc of serum (the titer was 10^{-1.3} for 0.03 cc), in view of the demonstration by Kitchen⁵ that the blood of sick mice contained at least 100 billion LD₅₀ of virus per cc. Another specimen of the patient's serum taken on the 5th day, coincident with the slight elevation in temperature, contained no virus but a definite, though small, amount of neutralizing antibody was already present (Table II).

R.W.B. first began working with the "K" virus on October 30, 1946 and because he felt feverish (temperature not taken) and had a moderately severe headache on the night of November 5, his blood taken on November 6 was inoculated in mice in the same manner and at the same time as that of A.B.S. However, no virus was recovered. The results of the neutralization tests against the "K" virus with the sera obtained from both A.B.S. and R.W.B., shown in Table II, indicate that only A.B.S. developed neutralizing antibodies and that the titer in his case had risen from 50 in the serum taken 4 days after onset to at least 1,585,000 in the serum taken 10 days later. The results of test B (Table II) are of especial interest because they suggest that after an initial high peak, the neutralizing antibody drops to a titer of about 1,000 and can then persist for at least 12 years without any further exposure to the virus in the interim. Both K.C.S.[†] and

T.F.[‡] suffered from accidental laboratory infections, and T.F. has had no further exposure to Rift Valley Fever virus since he stopped working with it in 1934.

Discussion. The clinical features and the duration of the febrile phase of the illness reported in this communication are no different from the human cases which resulted from exposure to lamb or early mouse-passage virus. Kitchen⁵ quotes a personal communication from G. M. Findlay regarding a human laboratory infection with virus which had undergone more than 400 successive passages in mice, the route and tissue used for passage not being indicated however; this case probably had a more protracted course than usual since it is stated that the virus was recovered from the blood on the 6th day of illness. The absence of any appreciable modification in the virulence of this virus for human beings by approximately 300 or more intracerebral passages in mice is to be contrasted with the rapid modification which occurs when dengue virus is passaged by this route in mice.⁸ The chances for modification or mutation would appear to be much less for a virus, such as that of Rift Valley Fever, which, without requiring any preliminary adaptation, multiplies readily in mice, than for a virus which, like that of dengue, can be made to propagate in mice only with the greatest difficulty. The disease produced by the virus of Rift Valley Fever has previously been compared to dengue,¹ sandfly fever¹ and influenza.^{4,5} However, one of us (A.B.S.) having spent several years studying both dengue and sandfly (pappataci) fever, finds that human infection with Rift Valley Fever virus is clinically indistinguishable from sandfly fever, but unlike the type of disease produced in the majority of human beings as a result of primary infection with various types of dengue virus. It has indeed been suggested¹ that what has in the past been diagnosed as 3-day fever or sandfly fever in certain parts of East Africa may very well be examples of Rift Valley Fever in man. Fortunately the differentiation is

[†] We are indebted to Dr. J. E. Smadel of the Army Medical School for this specimen of serum and to Dr. Kenneth C. Smithburn of the Rockefeller Foundation for the details of the case.

[‡] We are indebted to Dr. Thomas Francis, Jr., of the University of Michigan for the serum on this case (described in reference 6).

⁸ Sabin, A. B., and Schlesinger, R. W., *Science*, 1945, 101, 640, and unpublished observations.

easy in the laboratory since the blood of patients, with sandfly fever or dengue, inoculated intra-abdominally in mice, is without effect, while in the case of Rift Valley Fever the mice die in about 2 to 3 days.

Findlay⁹ has previously reported the persistence of neutralizing antibodies for the virus of Rift Valley Fever for 4 to 5 years in laboratory workers, who, however, might have had further exposures to the virus. The present demonstration of the persistence of the antibodies for a period of 12 years, is an unusual example of the duration of immunity after a single attack without further exposure to the virus during the intervening years.

Summary. 1. Accidental human labora-

⁹ Findlay, G. M., *Brit. J. Exp. Path.*, 1936, 17, 89.

tory infection with a strain of Rift Valley Fever virus which had undergone at least about 300 intracerebral passages in mice, indicated that no modification in its pathogenicity for man resulted from this mode of passage.

2. The virus was recovered from the blood 10 hours after the first appearance of headache but titration revealed that the virus content was very low—only 666 LD₅₀ per cc of serum. Four days after onset, no virus was detected in the blood, but neutralizing antibodies (index of 50) were already present and in the next 10 days increased to yield an index of at least 1,585,000.

3. Neutralizing antibodies for Rift Valley Fever virus (index of 3,160) were found 12 years after a single attack in the serum of an individual who had no further exposure to the virus during the intervening years.

15804 P

Streptomycin in the Therapy of Granuloma Inguinale.*

ROBERT B. GREENBLATT, HERBERT S. KUPPERMAN, AND ROBERT B. DIENST.
(Introduced by G. L. Kelly.)

From the University of Georgia School of Medicine, Augusta, Ga.

The use of antimonial preparations in the therapy of granuloma inguinale is well established. Its limitations, particularly in chronic cases, have long been known. Recurrences of the disease have occurred with a great degree of frequency even after apparent healing was attained. Other forms of treatment have been employed alone or in conjunction with the antimonials and have ranged from the local application of escharotic agents to surgery and x-radiation therapy. Although cure may be attained by adhering to one or another of these procedures, much is to be desired in the management of this minor venereal disease.

With the advent of the antibiotics it was hoped that a more adequate and specific

therapy for granuloma inguinale might be evolved. Preliminary observation with penicillin and tyrothricin showed that these drugs were of little value. One to 4 million units of penicillin administered for the expressed purpose of treating granuloma inguinale failed to control the granulomatous lesions. Tyrothricin was also without effect when it was applied locally in the treatment of granuloma inguinale.

Streptomycin, however, has proved so strikingly effective in the therapy of granuloma inguinale that a preliminary report is warranted at this time. A total of 23 patients has received streptomycin for granuloma inguinale. The diagnosis of the disease was established in all cases by demonstrating the presence of Donovan bodies in either spreads made from the lesion or by

* This study was aided by a grant from the United States Public Health Service.

histologic study of biopsy sections.[†] It is to be emphasized that no other form of treatment was instigated. The daily doses employed ranged from 0.3 to 1 g administered in 6 equal doses per 24 hours at 4-hour intervals. Total doses varied from 3.3 to 46 g given over a period of time extending from 6 to 46 days. The patients to whom the drug was administered may be divided into 3 groups: (1) untreated patients, (2) chronic cases inadequately treated over a period of years, (3) patients who had relapsed after an extensive course of fuadin or anthiomaline therapy.

Daily smears made from lesions after streptomycin injections showed that Donovan bodies could not be demonstrated 5 to 9 days after administration of the antibiotic. *Fusospirochetosis*, often observed as a superimposed infection, was controlled with but few exceptions within 48 to 72 hours. Healing followed a general pattern and was usually centripetal in effect. The extragenital lesions responded most rapidly to treatment. On the other hand, when moist, ulcerated areas were in constant contact with one another, as between the thigh and scrotum, response to therapy was slower. However, the Donovan bodies disappeared as rapidly in these recalcitrant lesions as in those that showed rapid clinical improvement. The lesions ultimately were covered by depigmented scar tissue.

Toxic reactions were noted in 2 patients.

[†] A note of appreciation is due Dr. Edgar R. Pund for the examination of these tissue sections.

One occurred in a colored male 29 years of age after 10 days of administration of a total dose of 6 g of streptomycin. The toxic manifestations were expressed as a febrile reaction associated with a maculo-papular rash of the extremities and malar regions of the face. In addition a fine vesicular eruption and edema of the lips were also observed. Pruritus accompanied the skin eruption. Fifty mg of benadryl administered twice a day failed to control or prevent the reaction from occurring when streptomycin was tried again. Another patient developed a mild burning of the conjunctiva also after 10 days of treatment with a total dose of 3 g of streptomycin. However, after discontinuing the drug for 4 days, resumption of the same daily dose failed to cause any untoward symptoms.

Follow-up examinations, while meager at present, indicate that relapses may occur. Two patients who had received less than 4 g of the drug returned with a recrudescence of the lesion. A third patient who had received 28 g. suffered a relapse several months later. Donovan bodies were readily demonstrable in the recurrent lesions of these three cases. More time must elapse before a true evaluation of the overall effectiveness of streptomycin can be established. Certainly the remarkable clinical improvement and the rapid disappearance of the Donovan bodies from the smears of patients are reassuring and indicate that streptomycin is the most effective agent available today for the treatment of granuloma inguinale.

Streptomycin in Treatment of Experimental Trypanosomiasis in White Mice and Chick Embryos.*

DONALD J. MERCHANT. (Introduced by M. H. Soule.)

From the Hygienic Laboratory, University of Michigan.

The striking results obtained by the use of streptomycin and penicillin, in certain bacterial diseases, has somewhat overshadowed their use against fungus and protozoan infections. With respect to trypanosomiasis, Neghme,¹ working with *T. cruzi* in mice, and Augustine, *et al.*,² investigating *T. lewisi* in rats, have reported completely negative results in the treatment of experimental trypanosomiasis with penicillin. More recently Schatz and colleagues³ have described an attempt to isolate an antibiotic active against *T. equiperidum*. In their work they found that a crude preparation of streptomycin immobilized the trypanosomes in freshly drawn rat blood but only when used in a concentration greater than 1:200. Anderson, Villela and Reed⁴ were unable to show cytolysis of *T. equiperidum* by penicillin or streptomycin; and subtilin, in their hands, failed to prolong the life of mice infected with this organism. The use of streptomycin in human trypanosomiasis, particularly Chagas disease, has been somewhat limited but from information available,⁵ the results have been regularly disappointing.

The following report presents the results of a study on the influence of streptomycin in experimental infections of mice and chick embryos with *T. brucei*, *T. equiperidum* and *T. hippicum*.[†] These flagellates are main-

tained in the laboratory stock culture collection by serial passage in guinea pigs. In the present study the blood of infected rats was used as the source of organisms.

Experimental Procedure. White Mice. White mice were inoculated subcutaneously with blood taken from the tail of an infected rat. Streptomycin was administered subcutaneously at 3-hour intervals, for a period of 4 days, beginning 24-36 hours after the preliminary inoculation. Total dosage ranged up to 16,000 units per mouse. Addinall⁶ gives the LD₅₀[‡] for mice as 750 mg per kg when given subcutaneously. Two control series were included in each experiment; (1) the trend of the infection in untreated mice and (2) the toxicity of the antibiotic for normal mice. The course of the disease was followed by making microscopical examinations of blood specimens taken, by snipping the tip of the tail, at 12- to 24-hour intervals. These examinations were made with the aid of dark-field illumination and the number of trypanosomes per oil immersion field (diameter 145 μ) was determined and recorded.

Chick Embryos. Fertile eggs, which had been incubated 10 days, were inoculated with heart's blood from an infected rat. The material was injected directly into the yolk sac using a 1½-inch, 20-gauge needle and entering from the air sac end of the egg. This

* Streptomycin, as the hydrochloride, was made available for this study through the courtesy of Merck and Co. and Abbott Laboratories.

¹ Neghme, Amador, *Science*, 1945, **101**, 115.

² Augustine, D. L., Weinman, D., and McAllister, J., *Science*, 1944, **99**, 19.

³ Schatz, A., Maguason, H. J., Waksman, S. A., and Eagle, H., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 143.

⁴ Anderson, H. H., Villela, G. G., and Reed, R. K., *Science*, 1946, **103**, 419.

⁵ Personal communication of Dr. M. H. Soule.

[†] The original sources of the trypanosome strains used in this study were: (1) *T. brucei*—lineally descended from the original isolation of Bruce (1896). Obtained from Dr. Thomas, Liverpool School of Tropical Medicine, about 1900. (2) *T. equiperidum*—from Dr. A. L. Tatum, University of Wisconsin. (3) *T. hippicum*—from Dr. Herbert Clark, Gorgas Memorial Institute, Ancon, Canal Zone.

⁶ Addinall, C. R., *The Merck Report*, October, 1945.

[‡] Lethal dose for 50% of mice treated.

TABLE I.
The Effect of Streptomycin on Various Trypanosome Infections in White Mice.

Days after inoculation	2	3	4	5	6	7	8	9
<i>T. brucei</i> + streptomycin	0/s*	1/f†	15/f	AC‡	∖§	Dead		
" " control	0/s	1/s	1/5f	10/f	∖	"		
<i>T. equiperidum</i> + streptomycin	1/s	1/10f	3/f	AC	AC	"		
" " control	1/s	1/10f	2/f	15/f	AC	"		
<i>T. hippicum</i> + streptomycin	0/s	0/s	1/s	∖	1/10f	10/f	25/f	Dead
" " control	0/s	0/s	1/s	∖	10/f	AC	AC	"

* 0/s = no trypanosomes present on slide.

† 1/f = 1 trypanosome per oil immersion field.

‡ AC = too numerous to count.

§ ∖ = no count made.

TABLE II.
Effect of Streptomycin on Various Trypanosome Infections in Chick Embryos.*

Days after inoculation	3	4	5	6	7	8	9
<i>T. brucei</i> + streptomycin	0/s	0/s	∖	1/10f	5/f	Dead	
" " control	0/s	0/s	∖	2/f	5/f	"	
<i>T. equiperidum</i> + streptomycin	0/s	1/s	2/f	3/f	15/f	∖	Dead
" " control	0/s	1/10f	5/f	10/f	15/f	∖	"
<i>T. hippicum</i> + streptomycin	0/s	∖	0/s	∖	1/s	1/f	"
" " control	0/s	∖	0/s	∖	1/10f	1/3f	"

* See footnotes to Table I.

hole was immediately sealed with sterile paraffin. The streptomycin was introduced through the same opening as the original inoculation after thorough application of tincture of iodine to the paraffin seal. Three treatments were given every 24 hours for a period of 4 days. The hole was resealed after each injection of streptomycin. The total dosage in several instances was 40,000 units. The same controls, *i.e.* normal infection and toxicity, were used as were included in the mouse series.

Samples of blood, for microscopic examination, were obtained by nicking the shell of the egg with a carborundum disc previously treated with tincture of iodine.⁷ The openings were made in a region rich in capillaries but not overlying the large vessels. The orifice was, in each case, carefully sealed with sterile paraffin. Uninfected embryos were subjected to the same treatment, sterile saline solution being substituted for the streptomycin. These embryos served to indicate damage due to trauma. The microscopic studies were made with the aid of dark-field illumination and the results recorded as previously described.

Results. Typical examples of the findings,

with the infection in mice, are presented in Table I. Each of the animals received 600 units of streptomycin at 8:30 a. m., 11:30 a. m., 2:30 p. m. and 5:30 p. m. and 1,600 units at 8:30 p. m. for a period of 4 days, making a total of 16,000 units for each mouse.

As shown by Table I, there was no significant difference in either the course or the duration of the trypanosome infection in treated and untreated mice. In certain other experiments, using *T. brucei*, a total quantity of 28,000 units of streptomycin per mouse was administered with the same results. The toxicity controls showed no ill effects from any of the quantities used.

Table II gives a typical example of the results of the experiments with chick embryos. Each embryo was treated with 2,000 units of streptomycin at 10:30 a. m. and 2:30 p. m. and 6,000 units at 5:30 p. m. for a period of 4 days, making a total of 40,000 units for each embryo.

The data in Table II clearly indicate that there was no significant difference in the course or duration of the infection between treated and untreated embryos. The toxicity controls survived and many hatched.

The data recorded in this series of experiments confirm the findings of Hood,⁷ who

⁷ Hood, Mary N., unpublished thesis.

made a thorough investigation of the course of infection of trypanosomiasis in animals and chick embryos with the same strains of organisms used in this study.

Summary. White mice and chick embryos were infected with *T. brucei*, *T. equiperidum* and *T. hippicum*. After a lapse of 24-36 hours streptomycin was administered subcutaneously to the mice and was injected into the yolk sac of the chick embryos. Each

mouse received a total of 16,000 units and each chick embryo, a total of 40,000 units of streptomycin. The course of the disease was followed by microscopic examination of blood specimens taken at regular intervals. As far as could be determined, this antibiotic agent did not alter the course of the infections or prolong the life of the treated mice or embryos.

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Low Toxicity of Sulfonamide Mixtures. II. Combinations of Sulfathiazole, Sulfadiazine and Sulfamerazine.*

DAVID LEHR.

From the Department of Pharmacology, New York Medical College, Flower and Fifth Avenue Hospitals, New York City.

A new and simple approach to the prevention of renal complications caused by sulfonamides was presented in a previous communication.¹ It consists in the use of mixtures of sulfonamides instead of single compounds. The idea emerged from the observation that a saturated aqueous or urinary solution of a sulfonamide could still be fully saturated with a second and third sulfonamide of different molecular structure, each compound behaving as though it were present alone and exerting no influence on the solubilities of the others. Consequently, in solutions containing several sulfonamides, the maximum obtainable concentration appeared expressed by the sum of the solubilities of all the drugs present. This finding applied to the free compounds, as well as to their acetylated homologues.

It was reasoned on the basis of this observation that the danger of intrarenal formation of sulfonamide crystals could be considerably reduced by employing combinations of partial dosages of 2 or more therapeutical-

ly equivalent sulfonamides instead of single compounds. The validity of this contention was demonstrated in experimental and clinical studies with a mixture of sulfathiazole and sulfadiazine.^{2,3}

The present paper deals with the toxicity of a sulfadiazine-sulfamerazine and a sulfathiazole-sulfadiazine-sulfamerazine combination. It contains also some additional data on the sulfathiazole-sulfadiazine mixture, in particular a comparative quantitative estimation of intrarenal sulfonamide deposits from sulfathiazole, sulfadiazine, and their combination.

Materials and Methods. Four hundred albino rats from our own colony, 8-12 weeks old and weighing between 160-210 g were employed in all experiments with the exception of the study on chronic toxicity in which weanling rats were used. The animals were kept on a standard diet (Rockland Farms Rat Diet) and had free access to water.

For determination of the acute toxicity, observation was continued for 5 days following a single intraperitoneal injection of the sulfonamides because of the well known de-

* This investigation has been aided by grants from the Josiah Macy, Jr., Foundation and the Schering Corporation, Bloomfield, N.J.

¹ Lehr, D., *Proc. Soc. Exp. Biol. and Med.*, 1945, 38, 11.

² Lehr, D., *J. Urol.*, 1946, 55, 548.

³ Lehr, D., Slobody, L. B., and Greenberg, W. B., *J. Pediat.*, 1946, 29, 275.

TABLE I.
Comparative Acute Toxicity in Male Albino Rats of Sodium Salts of Sulfadiazine (NaSD), Sulfathiazole (NaST), Sulfamerazine (NaSMD), and Mixtures of 2 and 3 of These Compounds.

Drug, g/kg body wt		Sulfonamide total amt	No. of animals	No. of dead	% dead	Died within days
Single intraperitoneal inj. of						
NaSD, 1.5	1.5	1.5	90	76	85	2-4
NaST, 1.1	1.1	1.1	25	16	65	1-4
NaSMD, 1.5	1.5	1.5	29	15	52	1-3
NaSD, 0.75 } NaST, 0.55 }	1.3	1.3	60	7	12	2-4
NaSD, 0.75 } NaSMD, 0.75 }	1.5	1.5	30	11	37	1-2
NaSD, 0.54 } NaST, 0.42 } NaSMD, 0.54 }	1.5	1.5	30	1	3	1-2

layed death from sulfadiazine, and to some extent also from sulfamerazine intoxication. As a rule 5% aqueous solutions of the sodium salts of sulfathiazole, sulfadiazine, and sulfamerazine were employed. Mixtures of two drugs were prepared conveniently by mixing equal parts of the 5% solutions so that the final concentration was 2.5% each of sulfadiazine and sulfathiazole, or sulfadiazine and sulfamerazine. If unequal amounts of 2 or 3 drugs were used, as in the acute toxicity study with the sulfathiazole-sulfadiazine mixture, the initial concentrations of the individual drugs were so adjusted, that mixing of equal fluid volumes resulted in the desired final concentrations of the 2 or 3 components. Thus identical amounts of fluid were injected in all groups. In each test the toxicity of combined sulfonamides was evaluated *simultaneously* with the toxicity of equal or comparable dosages of the separate components of the mixture.

Ten rats represented the minimum experimental unit. Subgroups of 5 animals each were placed into separate metabolism cages. The sulfonamide concentration in the blood was determined from the tail vein of each animal, at least at one occasion, in order to eliminate rats injected inadvertently into the gut instead of intraperitoneally. In some experiments the sulfonamide level in blood and urine was followed for 48 hours.

In other studies groups of animals were killed at predetermined intervals of a 48-

hour period. The blood was used for estimation of the sulfonamide and nonprotein nitrogen level. The entire urinary tract was carefully inspected for the presence of crystalline deposits. The kidneys were weighed and cross-sections examined under the lens for concrements in the pelvis and for intratubular drug precipitation. The sulfonamide concentration was then determined in the kidneys according to a procedure previously outlined in detail.⁴

The subacute toxicity was studied by repeated intraperitoneal injections of sublethal dosages of the sulfonamides and their mixtures. The level of nonprotein nitrogen and sulfonamide in the blood was determined repeatedly and the total daily drug excretion in the urine was followed throughout the entire experimental period.

The chronic toxicity was determined by incorporating the sulfonamides in various concentrations and combinations into the powdered form of the standard diet. The experiments were conducted with weanling rats weighing 60-80 g and planned for a period of 6 weeks. The food consumption was checked daily. The body weight of each animal was recorded twice weekly. Blood concentration and total urinary elimination of sulfonamide were determined at the same time intervals. The nonprotein nitrogen levels in the blood were estimated upon termination

⁴ Lehr, D., and Antopol, W., *Urol. and Cutan. Rev.*, 1941, 45, 545.

TABLE II.
Findings in Blood and Kidneys 24 Hours After a Single Intraperitoneal Injection of Sulfonamides in Albino Rats.

Findings in blood and tissues										
Group No.	Drug, g/kg	Blood			Kidney (moist tissue)					
		N.P.N., mg %	Total sulfa,		Wt. (both kidneys)	Total sulfa			% acetylation	Appearance of renal cross-section
			mg %	% acetylation		mg %	mg acetylation			
1	NaSD, 1.5	107	140	19	2287	454	10.3	23	Considerable amt of drug ppt. in entire medulla.	
2	NaST, 1.1	154	62	27	2137	160	3.4	30	Marked edema.	
3	NaSD, 0.75 } NaST, 0.55 }	104	30	7	1763	34	0.6	6	Same in the papilla and pelvis. Marked edema.	
4	NaSD, 0.75	143	51	22	2259	89	2.0	38	No ppt. visible in 2 animals. Trace of ppt. in 1 animal. Slight edema.	
5	NaST, 0.55	53	1	23	1436	0	0	0	Ppt. obstructs the papilla. Marked edema.	
6	Controls	48			1472				No ppt. visible. No edema.	
All figures represent the mean of the values from 3 female animals.										

All figures represent the mean of the values from 3 female animals.

of each experiment and correlated with the pathologic-anatomical findings in the kidneys.

In all experiments, the surviving rats were killed by exsanguination in ether narcosis. Postmortem examinations were performed on these animals and as far as feasible also on rats succumbing during an experiment. Special attention was given to changes in the urinary tract. The most important organs of representative animals were fixed in formaldehyde for histological study.

Determination of free and conjugated sulfonamide was carried out according to the method of Bratton and Marshall⁵ using a Klett Summerson photo colorimeter. All figures of drug concentration were expressed in terms of the free sulfonamides and not of their sodium salts. Obviously no differentiation could be made between sulfathiazole, sulfadiazine and sulfamerazine in determinations of mixtures in body fluids. However, these compounds have very similar molecular weights and, therefore, give almost identical colorimetric readings.

Results. 1. *Acute Toxicity.* A condensation of the most important results from the acute toxicity studies is presented in Tables I and II and in Fig. 1.

Sulfadiazine-Sulfathiazole Mixture. It is apparent from Table I that the extensive increase in the number of animals in the sulfathiazole-sulfadiazine experiment did not result in any significant change in the mortality figures as compared to those originally reported.¹

It was inferred from these results that the danger of renal blockage can be significantly reduced by the joint administration of sulfadiazine and sulfathiazole in *partial* dosages. Added proof for the validity of this viewpoint was derived from a series of experiments conducted to determine the accurate amounts of sulfonamide present in the kidneys 4 hr., 8 hr., 24 hr., and 48 hr. after a single intraperitoneal injection of the sulfadiazine-sulfathiazole mixture as compared to either compound when injected separately.

It was found that intrarenal precipitation

⁵ Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.

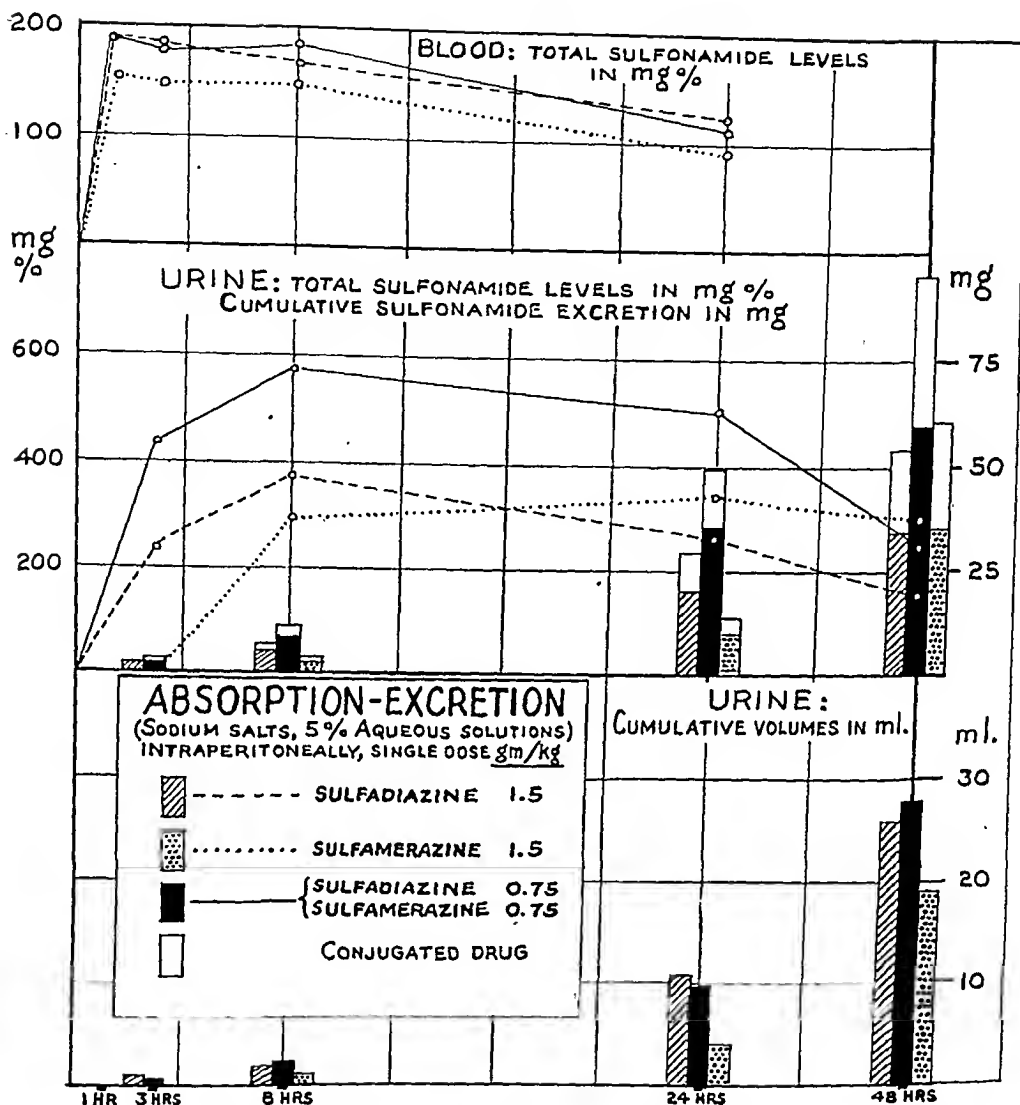


FIG. 1.

Graphic representation of the comparative absorption and excretion of sulfadiazine, sulfathiazole and a mixture of these two compounds in albino rats.

of sulfonamide crystals occurred initially in all groups, even in those receiving only *half* the dosages of either sulfathiazole or sulfadiazine alone. But there were highly significant differences in the quantity and duration of intratubular crystalline deposition.

Table II summarizes one representative experiment. The table demonstrates that after 24 hours the rats injected with the sulfadiazine-sulfathiazole mixture (Group 3) were practically free of renal drug deposits. Actual-

ly the kidneys of these animals contained less than one-tenth the amount of sulfonamide expected on the basis of the values from the full dosages of the separate compounds (*italic figures*), and even this small amount is fully accounted for by the general tissue concentration, as apparent from the sulfonamide blood level.

It was interesting to note that half the dose of sulfadiazine resulted in more precipitation when administered separately than

TABLE III.

Urinary Elimination of Single and Mixed Sulfonamides After One Intraperitoneal Injection in Albino Rats.

(Each value represents the mean from at least 10 animals.)

Drug	Dose, g/kg		% of administered dose excreted with urine in:		
	Partial	Total	8 hr	24 hr	48 hr
NaSD		1.5	1.9	9.6	16.0
NaSMD		1.5	1.2	5.4	24.0
NaSD	.75 }	1.5	4.7	21.0	40.0
NaSMD	.75 }				
NaSD	.54 }	1.5	9.0	39.0	50.0
NaSMD	.54 }				
NaST	.42 }				

TABLE IV.

Comparative Subacute Toxicity in Male Albino Rats of Sodium Salts of Sulfadiazine (NaSD), Sulfathiazole (NaST), Sulfamerazine (NaSMD), and Mixtures of 2 and 3 of These Compounds.

(The mortality figures are shown as they appeared when 100% mortality was reached in the sulfathiazole group, that is, after 4 injections.)

Drug, g/kg body wt		Sulfonamide total amt. inj.	No. of animals	% death	Died within days
Daily intraperitoneal inj. of					
NaSD, .9		3.6	15	87	2-5
NaST, .9		3.6	15	100	1-4
NaSMD, .9		3.6	20	55	3-5
NaSD, .45 }		3.6	10	30	3-4
NaST, .45 }					
NaSD, .45 }		3.6	10	0	
NaSMD, .45 }					
NaSD, .3 }		3.6	10	0	
NaST, .3 }					
NaSMD, .3 }					

when given in combination with sulfathiazole. This paradoxical phenomenon can be explained by the greater diuresis in the presence of higher sulfonamide concentrations in the tubular urine. It is obvious that sulfonamide mixtures retain the same osmotic value as equal concentrations of single compounds. Thus animals receiving the partial dose of only one component of the mixture are exposed to no less danger of oversaturation in the tubular urine, while at the same time they are deprived of the greater diuretic effect of higher sulfonamide concentrations as present in the urine of mixture animals.

Mixtures of Sulfadiazine-Sulfamerazine and Sulfadiazine-Sulfathiazole-Sulfamerazine. The low toxicity of these 2 combinations is

illustrated in Table I. It can be seen that a mixture of 3 sulfonamides is significantly less toxic than a mixture of 2 compounds.

The absorption and excretion of sulfonamide combinations is exemplified in Fig. 1. It is evident that a mixture of half dosages of sulfadiazine and sulfamerazine is more completely absorbed and excreted than either compound administered separately in the same total dosage. Similar observations were made with the mixture of sulfadiazine-sulfathiazole and the combination of all 3 sulfonamides. The latter gave the most complete urinary recovery figures.

In this connection, it should be kept in mind that excessive dosages of sulfonamides, which result in renal obstruction, are neces-

TABLE V. Body Weight, Nonprotein Nitrogen and Sulfonamide Levels in Blood, and Pathologic-Anatomical Findings in Kidneys of Albino Rats Surviving After 6 Weeks on a Diet Containing Either Sulfadiazine, Sulfamerazine, or a Mixture of the 2 Drugs.

% drug in the diet	No.	Sex	Body wt	Blood		Findings in the kidneys	Remarks
				N.P.N. mg %	Sulfonamide, mg %		
3% SMD	1	F	88	103	33	Parenchymatous damage in all survivors with calcifying nephrosis in 3 instances	1 died within 5 wk
	2	F	76	101	24		
	3	F	93	145	37		
	4	F	76	121	37		
	5	F	80	126	31		
	6	M	115				
	7	M	110	149	34		
	8	M	108	93	30		
	9	M	113	107	27		
	Avg		95	118	31		
3% SMD	2	F	63	89	56	Many concretions throughout entire urinary tract in one survivor; gravel in the pelvis and intrarenal precipitate in the other four	4 died within 4 wk
	5	F	76	103	57		
	8	M	84	110	53		
	9	M	60	84	69		
	10	M	80	64	54		
	Avg		73	90	58		
	1	F	78	70	64	Traces of gravel in 4 of 9 survivors; otherwise normal kidneys	1 died within 2 wk
	2	F	75	45	55		
	3	F	77	84	45		
	4	F	68	67	64		
	5	F	78	45	49		
	7	M	84	75	62		
	8	M	74	73	63		
	9	M	80	72	63		
	10	M	88	61	64		
	Avg		78	66	59		
Controls	1	F	148	53			
	2	F	144	47			
	3	F	166	44			
	4	F	150	46			
	5	F	160	34			
	6	M	248	45			
	7	M	202	58			
	8	M	192	54			
	9	M	200	46			
	10	M	215	50			
	Avg		183	48			

sarily much less completely eliminated with the urine than small amounts. Hence high recovery figures in the urine, as observed with sulfonamide combinations, can mean only diminished interference with renal elimination. In line with this interpretation, Table III indicates that the percentage of urinary recovery is directly proportional to the number of sulfonamides in the combination, since any addition to this number results necessarily in smaller partial dosages of each component.

2. *Subacute Toxicity.* The results of the studies in male albino rats are summarized in Table IV. All 3 sulfonamide combinations are again conspicuous because of their low toxicity.

A comparison with Table I reveals that the results of the subacute experiments were in good agreement with those of the acute study. This applied also to levels of non-protein nitrogen and sulfonamide in the blood as well as to urinary drug excretion and autopsy findings. Identical acute and subacute toxicity studies with *female* rats were confirmatory, although female animals proved in general distinctly more sensitive to the sulfonamides.

3. *Chronic Toxicity.* Studies with a sulfathiazole-sulfadiazine food mixture had to be abandoned because weanling rats submitted to almost complete voluntary starvation at sulfathiazole and at sulfathiazole-sulfadiazine concentrations of 5% and even 3% in the diet.

Sulfamerazine, although still less well tolerated than sulfadiazine, permitted an intake at the 3% level in the food sufficient both for the survival of the rats and for the creation of serious renal lesions. It should be stressed that at the same concentration the sulfadiazine food was consumed in larger amounts: accordingly, the voluntary intake of the sulfadiazine-sulfamerazine combination was in between the one of either compound given separately. The end result of a 6-week feeding study is shown in Table V.

Measured in terms of survival and inhibition of growth, sulfamerazine would appear to be more toxic than sulfadiazine and the

combination. In comparing sulfadiazine and the sulfadiazine-sulfamerazine mixture one might be misled by the greater retardation of growth from the latter into considering the mixture more toxic than sulfadiazine. However, if one compares the nonprotein nitrogen and sulfonamide levels in the blood as well as the findings in the kidneys, it becomes immediately apparent that sulfadiazine like sulfamerazine is by far more toxic than the combination of the 2 drugs.

The less significant growth inhibition in the sulfadiazine group was obviously due to the higher food consumption. Despite the simultaneously greater drug intake, the blood levels from sulfadiazine were throughout the experiment lower than from sulfamerazine and the combination. Since there was little difference in the total urinary excretion of sulfadiazine and sulfamerazine, it is obvious that sulfadiazine was slower and less completely absorbed from the gastro-intestinal tract and faster excreted by the kidneys than sulfamerazine. Renal damage was very marked in the sulfadiazine as well as in the sulfamerazine group, whereas it was insignificant in the animals fed the combination of the 2 drugs.

The fact that sulfamerazine may produce more concrement formation than a higher dose of sulfadiazine should serve as a warning that renal complications may occur from sulfamerazine at least as readily as from sulfadiazine. Although this finding is in contrast to previous experimental observations,^{6,7} it is strongly supported by growing clinical experience.^{8,9}

Discussion. The present study extended the experimental evidence for the strikingly low toxicity of sulfonamide combinations from the sulfathiazole-sulfadiazine mixture

⁶ Welch, A. D., Mattis, P. A., Latven, A. R., Benson, W. M., and Shields, E. H., *J. Pharm. and Exp. Therap.*, 1943, **77**, 357.

⁷ Schmidt, L. H., Hughes, H. B., and Badger, E. A., *J. Pharm. and Exp. Therap.*, 1944, **81**, 17.

⁸ Flippin, H. F., and Reinhold, J. G., *Ann. Int. Med.*, 1946, **25**, 433.

⁹ Hageman, P. O., Hartford, C. G., Sobin, S. S., and Ahrens, R. E., *J. A. M. A.*, 1943, **123**, 325.

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% drug in the diet	No.	Sex	Body wt.	Blood		Findings in the kidneys		Remarks
				N.P.N. mg %	Sulfonamide, mg %	Parenchymatous damage in all survivors with calcifying nephrosis in 3 instances		
3% S/D	1	F	88	103	33			
	2	F	76	101	24			
	3	F	93	145	37			
	4	F	76	121	37			
	5	F	80	126	31			
	6	M	115					
	7	M	116	149	34			
	8	M	108	93	30			
	9	M	113	107	27			
	Avg		95	118	31			
3% SMD	2	F	63	89	56	Many concretions throughout entire urinary tract in one survivor; gravel in the pelvis and intrarenal precipitate in the other four		
	5	F	76	103	57			
	8	M	84	110	53			4 died within 4 wk
	9	M	60	84	69			
	10	M	80	64	54			
	Avg		73	90	58			
	1	F	78	70	64			
	2	F	75	45	55			
	3	F	77	84	45			
	4	F	68	67	64			
1 1/2% S/D 1 1/2% SMD }	5	F	78	45	49	Traces of gravel in 4 of 9 survivors; otherwise normal kidneys		
	7	M	84	75	63			
	8	M	74	73	63			
	9	M	80	72	63			
	10	M	88	61	64			
	Avg		78	66	59			
	1	F	148	53				
	2	F	144	47				
	3	F	166	44				
	4	F	150	46				
Controls	5	F	160	34				
	6	M	248	45				
	7	M	202	58				
	8	M	192	54				
	9	M	200	46				
	10	M	215	50				
	Avg		183	48				

sarily much less completely eliminated with the urine than small amounts. Hence high recovery figures in the urine, as observed with sulfonamide combinations, can mean only diminished interference with renal elimination. In line with this interpretation, Table III indicates that the percentage of urinary recovery is directly proportional to the number of sulfonamides in the combination, since any addition to this number results necessarily in smaller partial dosages of each component.

2. *Subacute Toxicity.* The results of the studies in male albino rats are summarized in Table IV. All 3 sulfonamide combinations are again conspicuous because of their low toxicity.

A comparison with Table I reveals that the results of the subacute experiments were in good agreement with those of the acute study. This applied also to levels of non-protein nitrogen and sulfonamide in the blood as well as to urinary drug excretion and autopsy findings. Identical acute and subacute toxicity studies with *female* rats were confirmatory, although female animals proved in general distinctly more sensitive to the sulfonamides.

3. *Chronic Toxicity.* Studies with a sulfathiazole-sulfadiazine food mixture had to be abandoned because weanling rats submitted to almost complete voluntary starvation at sulfathiazole and at sulfathiazole-sulfadiazine concentrations of 5% and even 3% in the diet.

Sulfamerazine, although still less well tolerated than sulfadiazine, permitted an intake at the 3% level in the food sufficient both for the survival of the rats and for the creation of serious renal lesions. It should be stressed that at the same concentration the sulfadiazine food was consumed in larger amounts; accordingly, the voluntary intake of the sulfadiazine-sulfamerazine combination was in between the one of either compound given separately. The end result of a 6-week feeding study is shown in Table V.

Measured in terms of survival and inhibition of growth, sulfamerazine would appear to be more toxic than sulfadiazine and the

combination. In comparing sulfadiazine and the sulfadiazine-sulfamerazine mixture one might be misled by the greater retardation of growth from the latter into considering the mixture more toxic than sulfadiazine. However, if one compares the nonprotein nitrogen and sulfonamide levels in the blood as well as the findings in the kidneys, it becomes immediately apparent that sulfadiazine like sulfamerazine is by far more toxic than the combination of the 2 drugs.

The less significant growth inhibition in the sulfadiazine group was obviously due to the higher food consumption. Despite the simultaneously greater drug intake, the blood levels from sulfadiazine were throughout the experiment lower than from sulfamerazine and the combination. Since there was little difference in the total urinary excretion of sulfadiazine and sulfamerazine, it is obvious that sulfadiazine was slower and less completely absorbed from the gastro-intestinal tract and faster excreted by the kidneys than sulfamerazine. Renal damage was very marked in the sulfadiazine as well as in the sulfamerazine group, whereas it was insignificant in the animals fed the combination of the 2 drugs.

The fact that sulfamerazine may produce more concrement formation than a higher dose of sulfadiazine should serve as a warning that renal complications may occur from sulfamerazine at least as readily as from sulfadiazine. Although this finding is in contrast to previous experimental observations,^{6,7} it is strongly supported by growing clinical experience.^{8,9}

Discussion. The present study extended the experimental evidence for the strikingly low toxicity of sulfonamide combinations from the sulfathiazole-sulfadiazine mixture

⁶ Weleb, A. D., Mattis, P. A., Latven, A. R., Benson, W. M., and Shields, E. H., *J. Pharm. and Exp. Therap.*, 1943, **77**, 357.

⁷ Schmidt, L. H., Hughes, H. B., and Badger, E. A., *J. Pharm. and Exp. Therap.*, 1944, **81**, 17.

⁸ Flippin, H. F., and Reinhold, J. G., *Ann. Int. Med.*, 1946, **25**, 433.

⁹ Hageman, P. O., Harford, C. G., Sobin, S. S., and Ahrens, R. E., *J. A. M. A.*, 1943, **123**, 325.

to include combinations of sulfadiazine-sulfamerazine, and sulfathiazole-sulfadiazine-sulfamerazine. Confirmatory experimental results were reported recently by other investigators.^{10,11} In this connection it is of importance to stress that the toxicity of sparingly soluble sulfonamides is dependent upon 2 factors. The one, representing the true tissue toxicity, consists in the direct chemical action of the sulfonamides on the living cell; the other, as a function of solubility and renal clearance of these compounds, lies in the hazard of mechanical blockage in the urinary tract.

Lately the factors influencing the prevention and control of renal blockage from sulfonamides were summarized in a lucid review by Scudi.¹² The significance of renal obstruction in the over-all toxicity of heterocyclic derivatives of sulfanilamide was demonstrated in the animal experiment.^{4,13} It was also shown that measures which diminish the intrarenal formation of sulfonamide crystals¹⁴ or remove deposits from the tubules¹⁵ will result in a most significant drop in the mortality. The low toxicity of sulfonamide combinations can, therefore, be interpreted in the light of experimental proof for a strongly diminished tendency to intrarenal precipitation, as due mainly to a decrease in the renal factor of toxicity. This viewpoint is strengthened by the lack of evidence for any other mechanism of detoxification, since one would not expect pronounced changes in the true cellular toxicity of closely related compounds if they retain their full antibacterial activity in combinations.

In line with this interpretation was the further significant lowering of the toxicity for

a mixture of 3 sulfonamides. It was obviously due to the fact that the triple mixture contained necessarily *smaller* partial dosages of the individual compounds, resulting in a further diminution of the possibility of oversaturation and hence precipitation in the tubular urine.

Since at the bedside crystalluria was strongly decreased, even with mixtures of 2 sulfonamides and in the absence of alkalization,^{2,3,8} it seems justified to assume that one might safely dispense with the burden and disadvantages^{8,16} of adjuvant alkali therapy when employing combinations of 3 sulfonamides.

The unexpectedly high blood and urine levels obtained with mixtures, as well as the significantly greater recovery from the urine as compared to single sulfonamides, can be explained by a more complete absorption of mixtures and the greater ease of their renal elimination. These findings were confirmed in extensive absorption and excretion studies with sublethal dosages of sulfonamide combinations administered by the intraperitoneal and oral route. They proved that for any given sulfonamide the completeness of absorption and urinary elimination was, within limits, inversely proportional to the size of the dose used. This behavior remained unchanged for each drug if several different sulfonamides were administered simultaneously. In other words, *in mixtures of sulfonamides the body handles each compound as if it were present alone and in the amount contained in the combination.*

With regard to *in vitro* antibacterial activity, it was reported previously¹ that the sulfathiazole-sulfadiazine combination showed essentially an additive effect and that in some instances the combination was even more effective than either compound alone in equal concentration. Continuation of these experiments rendered identical results for a mixture of sulfadiazine and sulfamerazine, and a combination of all 3 compounds. However, it should be remembered that these observations were made in the test tube with equal sulfonamide concentrations, whereas

¹⁰ Whitehead, R., Sect. Exp. Med. and Therap., A.M.A. Meeting, San Francisco, July 3, 1946.

¹¹ Frisk, A. R., Hagerman, G., Helander, S., and Sjogren, B., *Nordisk Med.*, 1946, **29**, 639.

¹² Scudi, J. V., *Am. J. M. Sc.*, 1946, **211**, 615.

¹³ Lehr, D., Antopol, W., and Churg, J., *Science*, 1940, **92**, 434.

¹⁴ Lehr, D., *Bull. New York Acad. Med.*, 1944, **20**, 424.

¹⁵ Lehr, D., *Proc. Soc. Exp. Biol. and Med.*, 1944, **56**, 82.

¹⁶ Beyer, K. H., Peters, L., Patch, E. A., and Russo, H. F., *J. Pharmacol.*, 1944, **82**, 239.

the administration of equal dosages of the 3 sulfonamides and their combinations would result in greatly different drug concentrations in the living body. In accordance with the results of absorption-excretion studies, as exemplified in Fig. 1, sulfonamide mixtures give high and well sustained blood concentrations and should, therefore, prove at least of the same if not of higher antibacterial value when compared with the same dosage of any one of their individual components *in vivo*. Although this viewpoint is well supported by clinical experience,^{2,3} it remains to be accurately investigated in therapeutic studies with experimental infections of laboratory animals.

Summary. 1. In continuation of experimental and clinical studies with mixtures of sulfonamides, the toxicity as well as the absorption and excretion of the combinations sulfadiazine-sulfathiazole, sulfadiazine-sulfamerazine, and sulfadiazine-sulfathiazole-sulfamerazine were investigated in albino rats.

2. These combinations of partial dosages proved significantly less toxic than any one of their separate constituents in equal or comparable total dosage. The mixture of 3

sulfonamides was less toxic than either combination of 2 drugs.

3. The low toxicity of sulfonamide mixtures was shown to be due to the prevention of renal obstruction, resulting from a pronounced diminution in the intratubular deposition of sulfonamide crystals.

4. Mixtures of sulfonamides were more completely absorbed and excreted than equal amounts of their individual constituents. Blood levels from mixtures were, therefore, distinctly higher than expected on the basis of mathematical computations from the values of single sulfonamides.

5. It was reasoned on the basis of these experimental studies that the use of a mixture containing 3 sulfonamides in human therapy would almost completely eliminate the possibility of concrement formation in the urinary tract at the routine dose level. Hence, it would also obviate the necessity for adjuvant alkali therapy.

The technical assistance of the Misses Helen and Ruth Salzberg and Miss Catherine Russell is gratefully acknowledged.

15807

Surface Striations of *Euglena gracilis* Revealed by Electron Microscopy.

VINCENT GROUPÉ. (Introduced by Geoffrey Rake.)

From the Division of Microbiology, The Squibb Institute for Medical Research, New Brunswick, N.J.

In the course of studying a variety of microorganisms with the aid of electron microscopy using the shadow casting technic of Williams and Wyckoff¹ and the replica technic of Hillier and Baker² a definite pattern of surface striations on the pellicle of *Euglena gracilis* was clearly revealed. The presence of such surface striations on this

species suggests the possibility that many or all of the species of *Euglena* possess these markings in varying degree inasmuch as prominent surface striations have been described for other species of *Euglena* (e.g., *E. viridis*, *E. oxyuris*, and *E. spirogyra*).³

Bacteria-free cultures of 4 physiologically different species of *Euglena gracilis*, obtained through the courtesy of Dr. George W. Kidder, were maintained by serial passage on

¹ Williams, R. C., and Wyckoff, R. W. G., *Proc. Soc. Exp. Biol. and Med.*, 1945, **58**, 265.

² Hillier, J., and Baker, R. F., *J. Bact.*, 1946, **52**, 411.

³ Kudo, R. R., *Handbook of Protozoology*, Charles C. Thomas, Springfield, Ill., 1931, 117-120.

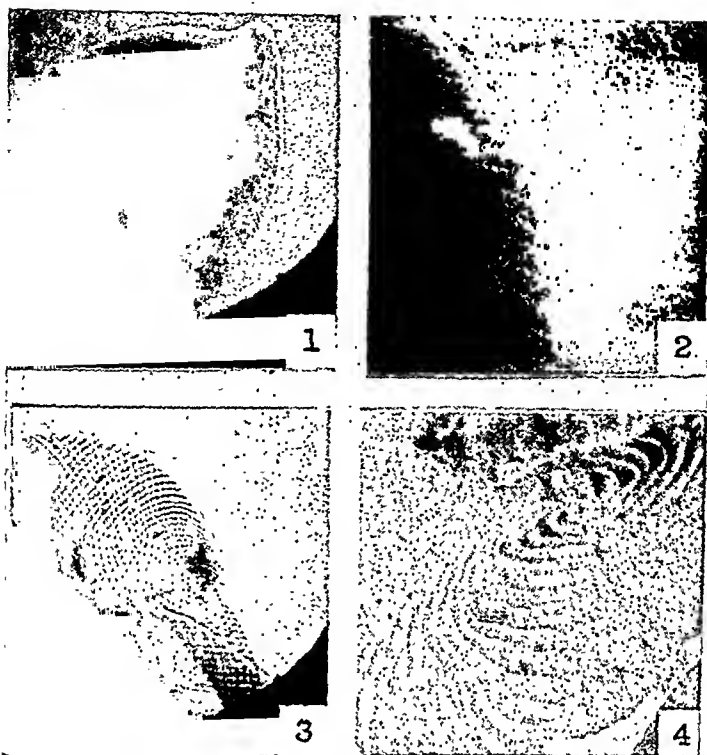


FIG. 1-4.

Electron Micrographs of *Euglena gracilis* Shadowed with Gold.

FIG. 1. Shadowed with 22.0 mg gold at the angle tangent $3/8.5$. Magnification 3370 \times .

FIG. 2. Shadowed with 22.0 mg gold at the angle tangent $3/8.5$. Magnification 7800 \times .

FIG. 3. Shadowed with 21 mg gold at the angle tangent $3/8.5$. Magnification 3370 \times .

FIG. 4. Shadowed with 24 mg gold at the angle tangent $3/9$. Magnification 8530 \times (collodion replica).

tryptone acetate broth. Organisms for study were obtained from plate cultures of the various strains on tryptone acetate agar and were prepared for examination in the electron microscope using the replica technic of Hillier and Baker.² Specimens thus prepared were shadowed with gold according to the technic of Williams and Wyckoff¹ using a shadow casting device designed and built by Dr. H. Sidney Newcomer. An RCA electron microscope (Type EMU) was used throughout these studies.

It will be seen from the pictorial data presented in Fig. 1 and 2 that groove-like striations forming a regular pattern are present

on the surface of *Euglena gracilis*. That these striations are located on the pellicle is evident from the micrograph presented in Fig. 3. It will be seen that the cell has ruptured allowing the protoplasm to escape and had left the pellicle virtually intact thus clearly revealing the pattern of striations on the membrane. Furthermore, it would appear that the striations originate from a central point located on the side of the pellicle and spiral outward and around the organism producing a pattern suggestive of a fingerprint. The structure of such a central point is shown in greater detail in the collodion replica of a portion of the surface of an organism pre-

sented in Fig. 4. A similar pattern of surface striations was observed on each of the 4 strains of *Euglena gracilis* studied.

Unfortunately, no intact flagella were observed and, although replicas of flagella were

occasionally encountered, no evidence of structural differentiation was seen.

Summary. A regular pattern of groove-like striations was observed on the pellicle of *Euglena gracilis*.

15808

In vitro Sensitivity of *Brucella* to Streptomycin: Development of Resistance During Streptomycin Treatment.*

WENDELL H. HALL AND WESLEY W. SPINK.

From the Division of Internal Medicine, University of Minnesota Hospitals and Medical School, Minneapolis.

Like many other species of bacteria brucella are remarkably sensitive *in vitro* to the action of streptomycin.¹⁻³ Experimental brucella infections in animals^{1,3} have also been favorably influenced by the administration of streptomycin. However, the use of streptomycin in human brucella infections has not been attended by consistently favorable results.⁴⁻⁸ The cause of this has not been elicited and there have been no reports in the literature of the development of resistance during the treatment of human brucella in-

fections with streptomycin. The present report summarizes the results of numerous tests of the *in vitro* sensitivity of the 3 varieties of brucella to streptomycin. Studies are reported concerning a strain of *Br. abortus* which developed marked resistance to the action of streptomycin during the treatment of a patient having subacute bacterial endocarditis due to this organism.

Methods and Materials. The method of testing the *in vitro* sensitivity of 40 strains of brucella was as follows: a loopful of a 24-hour culture grown on a Bacto tryptose phosphate agar[†] slant was transferred to 10 ml of sterile tryptose phosphate broth so as to give a suspension with a turbidity equal to that of a barium sulfate No. 1 standard. This suspension was then serially diluted to 10⁻³ with tryptose phosphate broth. Pour plate colony counts indicated that this diluted suspension contained 30,000 to 300,000 viable cells per ml. Each of a series of 10 test tubes was inoculated with 4.4 ml of tryptose phosphate broth. To each of 9 tubes was added 0.1 ml of the 10⁻³ dilution of brucella. The 10th tube was used as a sterility control. To all but the sterility control was added 0.5 ml of streptomycin dissolved in sterile physiological saline, the amount of streptomycin added being sufficient to give a final concentration of 0.5 to 10 μ g of streptomycin base per ml. The streptomycin solution

* This study was supported by grants from the American Medical Association and the United States Public Health Service and was carried out in the Laboratories of the Minnesota State Department of Health. The streptomycin was supplied by the Committee on Chemotherapeutic and Other Agents of the National Research Council.

¹ Jones, D., Metzger, H. J., Schatz, A., and Waksman, S. A., *Science*, 1944, **100**, 103.

² Waksman, S. A., and Schatz, A., *J. Am. Pharm. A. (Scient. Ed.)*, 1945, **34**, 237.

³ Live, I., Sperling, F. G., and Stubbs, E. L., *Am. J. M. Sc.*, 1946, **211**, 267.

⁴ Herrell, W. E., and Nichols, D. R., *Proc. Staff Meet., Mayo Clin.*, 1945, **20**, 449.

⁵ Reimann, H. A., Price, A. H., and Elias, W. F., *Arch. Int. Med.*, 1945, **76**, 269.

⁶ Nichols, D. R., and Herrell, W. E., *J. A. M. A.*, 1946, **132**, 200.

⁷ Keefer, C. S., *J. A. M. A.*, 1946, **132**, 4.

⁸ Hall, W. H., Braude, A., and Spink, W. W., *Staff Meet. Bull. Hosp. of Univ. of Minn.*, 1946, **18**, 109.

[†] Difco Laboratories, Detroit.

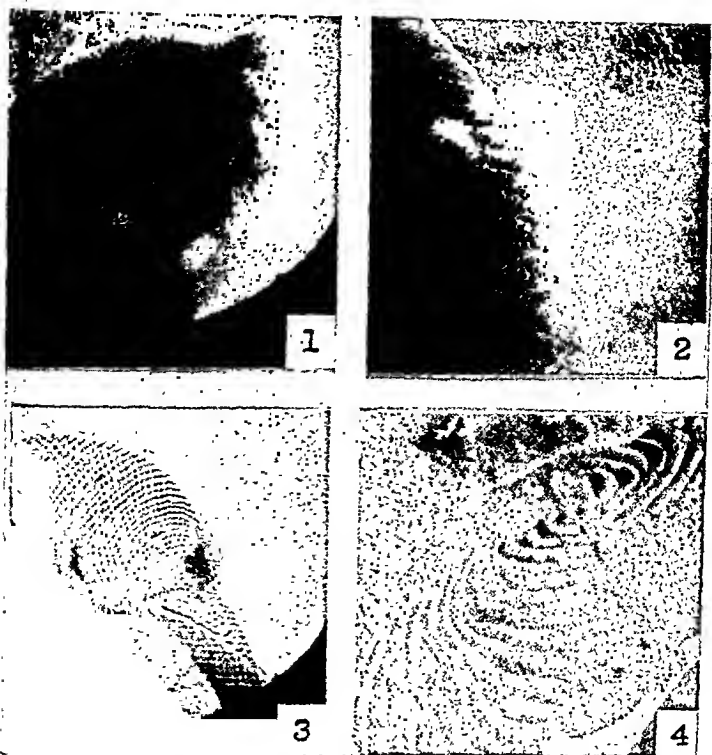


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TABLE II.
Effect of Carbon Dioxide on *in vitro* Sensitivity of
Brucella to Streptomycin.

Variety of <i>brucella</i> and strain	Minimum concentration of streptomycin completely inhibiting growth, $\mu\text{g/ml}$	
	Aerobic conditions	10% CO ₂ added
<i>Brucella abortus</i>		
439	1	2
552	0.5	1
<i>Brucella suis</i>		
37	2	3
80	2	3
88	2	3
145	1	3
616	1	3

inoculum affected the concentration of streptomycin necessary to completely inhibit growth *in vitro*. The growth of strain 524 of *Br. abortus* was completely inhibited by 1 μg per ml when an inoculum of 30,000 cells was used but 4 μg per ml was necessary when the inoculum was 30,000,000 cells. The concentration of streptomycin necessary to completely inhibit the growth of brucella is also dependent upon the time factor. One loop subcultures taken at 24-hour intervals revealed that after 48 hours exposure to streptomycin 15 of 20 strains of *Br. abortus* were inhibited by a concentration of streptomycin equal to 50% of that necessary to inhibit their growth after only 24 hours exposure. Subcultures at the end of 3 and 7 days revealed no further apparent increase in sensitivity. Similar observations were made with 3 of 7 strains of *Br. suis* and one strain of *Br. melitensis*. Furthermore there was no evidence that prolonged incubation of brucella in contact with streptomycin promoted the growth of streptomycin-resistant variants.

It is of interest that in 2 patients with chronic brucellosis and bacteremia who failed to benefit from streptomycin therapy there was no evidence that this failure was due to the development of streptomycin-resistant organisms. Nor was there any evidence that the presence of human serum reduced the *in vitro* sensitivity of these strains of *Br. abortus* to streptomycin. The first of these patients (P.L.) received 19 g of streptomycin intramuscularly in 9 days. Strain 424,

Br. abortus, was isolated from her blood stream before treatment and proved to be sensitive to 2 μg of streptomycin per ml. Strain 524 was isolated from her blood after 8 days of streptomycin therapy and was equally sensitive. The second patient (A.S.) was given 27.6 g of streptomycin intramuscularly in 9 days. Strain 483, *Br. abortus*, was recovered from his blood before streptomycin therapy and was inhibited by 1.5 μg streptomycin per ml. Four months later strain 11-5 was isolated from his blood, and it showed no decrease in streptomycin sensitivity.

However, in a third patient (K.E.) streptomycin resistance did develop during streptomycin therapy. This patient was a young farmer who developed acute brucellosis in 1944. His symptoms remitted with sulfonamide and vaccine therapy. However, his symptoms recurred 2 years later and were accompanied by signs of subacute bacterial endocarditis. *Br. abortus* strain 9-28 was isolated from his blood culture; it proved to be sensitive to 1.0 μg streptomycin per ml. He was then given 118 g of streptomycin intramuscularly over a period of 31 days. Strain 11-6 was isolated from his blood on the 29th day of streptomycin therapy. This strain grew in the presence of 7,500 μg of streptomycin per ml but its growth was inhibited in the presence of 10,000 μg per ml. *Br. abortus* was isolated from his blood 4 and again 5 days later; these cultures were equally resistant to streptomycin *in vitro*. One loop subcultures revealed the presence of a few viable brucella when these resistant cultures were exposed for 24 hours to concentrations as high as 50,000 μg of streptomycin per ml *in vitro*. The patient was given large doses of sulfadiazine orally for 2½ months at the conclusion of his streptomycin therapy and at the present time his infection is apparently arrested.

The streptomycin-resistant strain differed from strain 9-28 in several particulars. It grew much more slowly on tryptose phosphate agar and in tryptose phosphate broth. Its growth was particularly slow in the depth of tryptose phosphate agar pour plates, colonies not being visible until the end of 8 to 11

was preserved in the frozen state in a dry ice freezing cabinet and showed no decline in activity as a result of storage for several weeks. Commercial streptomycin in the form of the sulfate or hydrochloride from several different producers was used. Each lot of streptomycin was checked for potency against a sensitive strain of *Br. abortus*. After the addition of the streptomycin each tube was mixed thoroughly by rotation. The tubes were then incubated at 37°C. Unless otherwise stated the strains of *Br. abortus* were incubated under 10% CO₂. After 24 hours incubation each tube was mixed and one standard loopful. was subcultured on a tryptose phosphate agar plate. The plates were incubated at 37°C (*Br. abortus* under 10% CO₂) and examined for colonies of brucella with a hand lens after 96 hours.

The majority of the brucella cultures were tested for streptomycin sensitivity within a few days after they had been isolated from the blood of patients. In some cases the cultures had been suspended in 10% horse serum and stored in the frozen state for several months. After a few transfers on tryptose phosphate agar each culture was tested to ascertain that it was smooth and had the biochemical characteristics of one of the 3 varieties of brucella. We had the opportunity of testing the sensitivity to streptomycin of cultures of *Br. abortus* isolated from the blood of 3 patients before, during and/or after streptomycin therapy.

Results. In Table I are given the concentrations of streptomycin which completely inhibited the growth of 26 cultures of *Br. abortus*, 13 cultures of *Br. suis*, one stock culture of *Br. melitensis* (of unknown origin) and one stock rough culture of *Br. abortus*. None of the patients had received streptomycin therapy prior to the isolation of the organisms. Inspection of Table I reveals that all the strains of brucella were very sensitive to the action of streptomycin *in vitro* with the exception of the rough stock culture of *Br. abortus*. At first glance strains of *Br. suis* appear to be more sensitive than most strains of *Br. abortus*. The difference lies in the fact that incubation under an atmosphere containing 10% carbon dioxide re-

TABLE I.
Sensitivity of *Brucella* to Streptomycin *in vitro*.

Variety of brucella, and strain	Max. conc. permitting growth, µg/ml	Min. conc. completely inhibiting growth, µg/ml
<i>Brucella abortus</i>		
Stock	0.5	1
83	1	2
165	—	0.5
94	2	3
98	1	2
104	2	3
110	2	3
132	1	2
372	1	2
419	1	2
424	1	2
439	1	2
449	1	2
467	2	3
483	1	1.5
484	3	4
488	0.5	2
490	2	3
495	1	2
524	0.5	2
552	0.5	1
607	1	2
Grono (Rough)	50	100
Rowe 11-9	0.5	1
Shadiek 11-5	1	1.5
Elsted 9-28	0.5	1
<i>Brucella suis</i>		
Stock	1	2
67	0.5	1
156	1	2
263	1	2
303	0.5	1
306	0.5	1
346	0.5	1
456	0.5	1
37	2	3
80	1	2
88	1	2
145	0.5	1
616	0.5	1
<i>Brucella melitensis</i>		
Stock	1	2

duces the sensitivity of brucella to streptomycin. Strains of *Br. abortus* which had been adapted to aerobic growth increased in sensitivity to streptomycin when the test was carried out aerobically. When strains of *Br. suis* were tested for streptomycin sensitivity in an atmosphere containing 10% carbon dioxide streptomycin proved to be less effective in inhibiting their growth. The magnitude of the difference is shown in Table II.

It was also found that the size of the

Serological Groups and Types of Hemolytic Streptococci Isolated in Puerto Rico.*

A. POMALES-LEBRÓN, P. MORALES-OLIVERO, AND J. BARRETT

From the Department of Bacteriology of the School of Tropical Medicine, San Juan, P. R.

One hundred and sixteen pairs of tonsils were studied bacteriologically. The purulent exudate from the crypts and the inside of each pair of tonsils were cultured separately utilizing methods previously described¹ and the hemolytic streptococci obtained from these tonsils were studied serologically. The distribution of these cultures among Lancefield's groups is given in Table I. The results of a similar study of hemolytic streptococci from normal throats,² carried out in 1943-1944, is also included in Table I for purposes of comparison.

The tonsils yielded 62.8% cultures of β -hemolytic streptococci as compared with 17.4% positives among the normal throats. This difference was due mainly to a much higher percentage of Group A organisms obtained from tonsils (30.2%) than from normal throats (1.2%). The proportion of tonsil strains belonging to groups other than A was only twice as large (32.6%) as that obtaining among the throat cultures (16%).

Group B streptococci were obtained in 6% of the tonsils examined. No Group B strains were isolated from the normal throats, but there exists the possibility of these being overlooked if present in sparse numbers.

The distribution of the different Lancefield's groups among the aerobic nonlactose flora is given in some detail in Table II.

When the distribution of strains of different Lancefield's groups cultured from tonsils was compared with the distribution of strains

previously obtained from normal throats,² it was observed that Group A organisms predominated abundantly in 21 (60%) of the tonsils from which Group A strains were cultured. Contrasting markedly with this, no Group A strain were found in large number among the throats from which A organisms were cultured.

Group C organisms were abundant in 4 (57.1%) of tonsils from which C strains were obtained and in 26 (36.1%) of the normal throats from which C streptococci were isolated.

Group G organisms were obtained in large numbers in 4 (23.4%) and in 5 (14%) respectively of the tonsils and normal throats from which G organisms were cultured.

These figures may suggest that hemolytic streptococci of Groups G and C are of doubtful significance in the production of tonsillitis, however, streptococci belonging to these serological groups are occasionally obtained in abundant numbers and in pure or practically pure culture from diseased tonsils in the absence of any other common pathogen.

No conclusions can be drawn concerning Group B and F due to the small number of strains. It is of interest, however, to observe that these streptococci can be occasionally obtained in pure culture from the pits in the crypts and the inside of tonsils.

Our results agree closely with those reported by Kozak et al.³ in a similar study carried out in Melbourne, Australia.

The serological typing of 95 strains of Group β streptococci obtained from diseased

* This work has been possible through a grant of the Sanchez Department of Agriculture.

¹ Pomales-Lebrón, A., P. Morales-Oliviero, and J. Barrett, *Ann. N. Y. Acad. Sci.*, 22, 1946.

² Pomales-Lebrón, A., Morales-Oliviero, P., and Barrett, J., *Ann. N. Y. Acad. Sci.*, 22, 1946.

³ Kozak, R. J., Johnston, J. J., and Barrett, J., *Ann. N. Y. Acad. Sci.*, 22, 1946.

⁴ Kozak, R. J., Barrett, J. J., and Johnston, J., *Ann. N. Y. Acad. Sci.*, 22, 1946.

days of incubation. Two distinct colony forms were noted: (1) A large colony type growing relatively rapidly, producing colonies 2 to 3 mm in diameter in 48 hours. These colonies had a smooth surface and were very sensitive to the bactericidal action of human serum but grew in as much as 50,000 μg of streptomycin per ml. Gram stains revealed typical coccobacilli of uniform size and eosinophilic staining properties. They did not ferment the usual sugars and were agglutinated to a high titer by anti-brucella rabbit serum. This colony type formed only a small percentage of the total bacterial population. (2) A small colony form was much more numerous. Minute colonies 0.1 mm in diameter appeared in large numbers 4 to 5 days after the resistant strain was streaked on the surface of a tryptose phosphate agar slant. They died quickly unless the inoculum was heavy. They were relatively resistant to the bactericidal action of human serum but would not grow *in vitro* in the presence of more than 7,500 μg of streptomycin per ml. Gram stains revealed a mixture of bizarre, tiny, amorphous, coccoid organisms staining only faintly red and large, dark red, coccoid forms. The small colony variant also had all the serological and biochemical characteristics of *Br. abortus*. Both colony types were smooth and grew only in the presence of added carbon dioxide.

The resistance of strain 11-6 to streptomycin has not changed over a period of 3 months; during this period it has been transferred frequently on tryptose phosphate agar slants. This strain was streaked across the surface of a tryptose phosphate agar plate containing a heavy inoculum of sensitive *Br. abortus*, strain 524 and 3 μg streptomycin per ml. No satellite colonies of strain 524 appeared adjacent to the growth of the streptomycin-resistant strain. It was concluded that the resistant strain did not produce an extracellular, soluble, diffusible streptomycin inhibitor. The apparent resistance of this strain *in vitro* decreased as the length of exposure to streptomycin was increased. Thus subcultures at the end of 7 days incubation revealed that the large colony form was inhibited by 2,500 μg per ml but the small

colony variant remained resistant to 7,500 μg per ml. It is of interest that the *in vitro* growth of the resistant strain was stimulated by the addition of sublethal concentrations of commercial streptomycin. The stimulation of growth was particularly noticeable with concentrations of 50 to 1,000 μg of streptomycin per ml. This phenomenon was observed in tryptose phosphate broth, meat infusion broth, tryptose phosphate agar and human serum. The growth of the small colony form appeared to be stimulated more than that of the large colony form. No conclusive evidence was obtained indicating that any similar phenomenon occurs when sensitive strains of *Br. abortus* were exposed to sublethal concentrations of streptomycin.

Discussion. With the exception of one rough strain, all the brucella cultures tested proved to be very sensitive *in vitro* to streptomycin. Brucella appear to develop streptomycin resistance *in vivo* only after prolonged streptomycin treatment. The situation is analogous to that which obtains with *M. tuberculosis* and is presumably due to the slow rate of growth of brucella. It does not appear that the development of resistance to streptomycin by brucella can be the explanation for the frequent therapeutic failures which have been observed after relatively short periods of streptomycin therapy in human brucellosis. While better therapeutic results might follow more extended periods of streptomycin therapy, the possibility of the ensuing development of streptomycin-resistant strains of brucella must also be considered. It is reassuring however that one such streptomycin-resistant strain of *Br. abortus* was less refractory to the bactericidal action of human blood than its sensitive progenitor and remained sensitive to sulfadiazine.

Summary. 1. A method for the *in vitro* testing of sensitivity of brucella to streptomycin is described. 2. The streptomycin sensitivity of 40 cultures of brucella isolated from humans is given. 3. Studies are reported concerning a strain of *Br. abortus* which developed marked resistance to streptomycin during the treatment of a patient having subacute bacterial endocarditis.

Serological Groups and Types of Hemolytic Streptococci Isolated in Puerto Rico.*

A. POMALES-LEBRÓN, P. MORALES-OTERO, AND J. BARALT.

From the Department of Bacteriology of the School of Tropical Medicine, San Juan, P.R.

One hundred and sixteen pairs of tonsils were studied bacteriologically. The purulent exudate from the crypts and the inside of each pair of tonsils were cultured separately utilizing methods previously described¹ and the hemolytic streptococci obtained from these tonsils were studied serologically. The distribution of these cultures among Lancefield's groups is given in Table I. The results of a similar study of hemolytic streptococci from normal throats,² carried out in 1943-1944, is also included in Table I for purposes of comparison.

The tonsils yielded 62.8% cultures of β -hemolytic streptococci as compared with 17.4% positives among the normal throats. This difference was due mainly to a much higher percentage of Group A organisms obtained from tonsils (30.2%) than from normal throats (1.2%). The proportion of tonsil strains belonging to groups other than A was only twice as large (32.6%) as that obtaining among the throat cultures (16%).

Group B streptococci were obtained in 6% of the tonsils examined. No Group B strains were isolated from the normal throats, but there exists the possibility of these being overlooked if present in sparse numbers.

The distribution of the different Lancefield's groups among the aerobic tonsillar flora is given in some detail in Table II.

When the distribution of strains of different Lancefield's groups cultured from tonsils was compared with the distribution of strains

previously obtained from normal throats,² it was observed that Group A organisms predominated abundantly in 21 (60%) of the tonsils from which Group A strains were cultured. Contrasting markedly with this, no Group A strain were found in large number among the throats from which A organisms were cultured.

Group C organisms were abundant in 8 (57.1%) of tonsils from which C strains were obtained and in 26 (36.1%) of the normal throats from which C streptococci were isolated.

Group G organisms were obtained in large numbers in 4 (28.4%) and in 5 (16%) respectively of the tonsils and normal throats from which G organisms were cultured.

These figures may suggest that hemolytic streptococci of Groups G and C are of doubtful significance in the production of tonsillitis, however, streptococci belonging to these serological groups are occasionally obtained in abundant numbers and in pure or practically pure culture from diseased tonsils in the absence of any other common pathogen.

No conclusions can be drawn concerning Group B and F due to the small number of strains. It is of interest, however, to observe that these streptococci can be occasionally obtained in pure culture from the pus in the crypts and the inside of tonsils.

Our results agree closely with those reported by Keogh *et al.*³ in a similar study carried out in Melbourne, Australia.

The serological typing of 95 strains of Group A streptococci obtained from excised

* This work has been possible through a grant of the insular Department of Agriculture.

¹ Pomales-Lebrón, A., *P. R. J. Pub. Health and Trop. Med.*, 1929, **2**, 196.

² Pomales-Lebrón, A., Damin, G. J., Pons, C., and Morales-Otero, P., *P. R. J. Pub. Health and Trop. Med.*, 1946, 287.

³ Keogh, E. V., MacDonald, I., Battle, J., Simmons, R. R., and Williams, S., *British Med. J.*, 1939, **2**, 1036.

⁴ Swift, H. F., Wilson, A. T., and Lancefield, R. C., *J. Exp. Med.*, 1943, **78**, 127.

days of incubation. Two distinct colony forms were noted: (1) A large colony type growing relatively rapidly, producing colonies 2 to 3 mm in diameter in 48 hours. These colonies had a smooth surface and were very sensitive to the bactericidal action of human serum but grew in as much as 50,000 μg of streptomycin per ml. Gram stains revealed typical coccobacilli of uniform size and eosinophilic staining properties. They did not ferment the usual sugars and were agglutinated to a high titer by anti-*Brucella* rabbit serum. This colony type formed only a small percentage of the total bacterial population. (2) A small colony form was much more numerous. Minute colonies 0.1 mm in diameter appeared in large numbers 4 to 5 days after the resistant strain was streaked on the surface of a tryptose phosphate agar slant. They died quickly unless the inoculum was heavy. They were relatively resistant to the bactericidal action of human serum but would not grow *in vitro* in the presence of more than 7,500 μg of streptomycin per ml. Gram stains revealed a mixture of bizarre, tiny, amorphous, coccoid organisms staining only faintly red and large, dark red, coccoid forms. The small colony variant also had all the serological and biochemical characteristics of *Br. abortus*. Both colony types were smooth and grew only in the presence of added carbon dioxide.

The resistance of strain 11-6 to streptomycin has not changed over a period of 3 months; during this period it has been transferred frequently on tryptose phosphate agar slants. This strain was streaked across the surface of a tryptose phosphate agar plate containing a heavy inoculum of sensitive *Br. abortus*, strain 524 and 3 μg streptomycin per ml. No satellite colonies of strain 524 appeared adjacent to the growth of the streptomycin-resistant strain. It was concluded that the resistant strain did not produce an extracellular, soluble, diffusible streptomycin inhibitor. The apparent resistance of this strain *in vitro* decreased as the length of exposure to streptomycin was increased. Thus subcultures at the end of 7 days incubation revealed that the large colony form was inhibited by 2,500 μg per ml but the small

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Discussion. With the exception of one rough strain, all the *Brucella* cultures tested proved to be very sensitive *in vitro* to streptomycin. *Brucella* appear to develop streptomycin resistance *in vivo* only after prolonged streptomycin treatment. The situation is analogous to that which obtains with *M. tuberculosis* and is presumably due to the slow rate of growth of *Brucella*. It does not appear that the development of resistance to streptomycin by *Brucella* can be the explanation for the frequent therapeutic failures which have been observed after relatively short periods of streptomycin therapy in human brucellosis. While better therapeutic results might follow more extended periods of streptomycin therapy, the possibility of the ensuing development of streptomycin-resistant strains of *Brucella* must also be considered. It is reassuring however that one such streptomycin-resistant strain of *Br. abortus* was less refractory to the bactericidal action of human blood than its sensitive progenitor and remained sensitive to sulfadiazine.

Summary. 1. A method for the *in vitro* testing of sensitivity of *Brucella* to streptomycin is described. 2. The streptomycin sensitivity of 40 cultures of *Brucella* isolated from humans is given. 3. Studies are reported concerning a strain of *Br. abortus* which developed marked resistance to streptomycin during the treatment of a patient having subacute bacterial endocarditis.

TABLE III.
Typing of Hemolytic Streptococci from Different Sources.

Typing of Hemolytic Streptococci from Different Sources.															
Source	No. of strains tested	No. and %		Types										Cross reacted*	Negative with serums tested
		44	1	4	41	19	39	33	14	36					
Excised tonsils	33	No. 8	2	2	2	1	0	0	0	0	0	3	15		
		% 24.3	6.1	6.1	6.1	3.0	0	0	0	0	0	9.1	45.5		
Pathological throats, abscesses, ear and skin infections	22	No. 1	0	0	3	1	1	1	0	0	0	4	11		
		% 4.5	0	0	13.7	4.5	4.5	4.5	0	0	0	18.2	50		
From lesion or affected limb. Recurrent lymphangitis	9	No. 1	0	0	2	0	0	1	0	0	0	0	5		
		% 11.1	0	0	22.2	0	0	11.1	0	0	0	0	55.5		
Normal throats	31	No. 2	3	0	1	3	0	1	1	1	1	1	18		
		% 6.4	9.7	0	3.2	9.7	0	3.2	3.2	3.2	3.2	3.2	58.1		
Totals	95	No. 12	5	2	8	5	1	3	1	1	1	8	49		
		% 12.6	5.3	2.1	8.4	5.3	1	3.2	1	1	1	8.4	51.6		

* One strain gave a slight precipitate with types 3, 5, 22, 29, 31. Negative with the others. One culture gave a precipitate with No. 28 and No. 44 type serums. The other 6 cross reacting strains gave very slight precipitates with the majority of the type serums.

conclusions can be drawn concerning the distribution of the different types among the various sources.

It must be noted that approximately one-half our strains were untypable. Swift *et al.*⁴ state: "negative reactions may also be due to loss of type-specific M substance after isolation of the strain. In some strains and types the M substance remains constant, but in others it may disappear rapidly during subculture on artificial media." The extracts of the strains from excised tonsils were made from stock cultures kept on blood agar slants for many months. The fact that approximately the same proportion of strains from different sources were untypable suggests that this failure was not mainly due to degradation *in vitro* of the organisms with loss of the M substance. There exists the possibility that these strains may be capable of losing their M substance *in vivo*, or may belong to types other than those for which they were tested.

Summary. A bacteriological study of 116 pairs of excised tonsils showed that 62.8% harbored β -hemolytic streptococci. In contrast 705 cultures of normal throats similarly studied showed an incidence of 17.4% of this organism. This difference is due mainly to a higher percentage of Group A streptococci (30.2%) found in excised tonsils as compared to 1.2% in normal throats.

The proportion of streptococci cultured from excised tonsils belonging to groups other than A was twice as large (32.6%) as that obtained from normal throat cultures (16%). In typing 95 strains of hemolytic streptococci by the precipitin method described by Swift *et al.*⁴ representatives of types 44, 41, 1, 14, 19, 4, 36 and 39 were found. Approximately half the strains were untypable with the serums tested. Types 44 and 41 were most commonly found.

† We are greatly indebted to Dr. R. C. Laucefield for supplying type serums and homologous extracts for this study. Extracts from each of our strains were tested with the following type serums, 1, 2, 3, 4, 5, 6, 8, 9, 11, 12, 13, 14, 15, 16, 17, 18, 19, 22, 23, 24, 26, 28, 29, 30, 31, 32, 33, 36, 37, 38, 39, 41, 43, 44, and 46.

TABLE I.
Hemolytic Streptococci from 116 Pairs of Excised Tonsils and from 705 Normal Throats.

Tonsils.							
No. and %	Groups					Total positive	Negative
	A	B	C	G	F		
No. positive	35	7	14	14	3	73	43
% "	30.2	6	12	12	2.6	62.8	(37.2%)
Normal Throats.							
No. "	9	0	72	42	1	123	582
% "	1.2	0	10.2	6.0	1	17.4	(82.6%)

TABLE II.
Distribution of Streptococci of Groups A, B, C, G, and F (Lancefield) Among the Aerobic Flora of Excised Tonsils.

Distribution	Cases in which this distribution occurred	
	No.	%
Group A		
Abundantly predominating or in pure culture	21	60.0
Numerous colonies, abundant growth <i>Staph. aureus</i>	3	8.6
Few colonies, <i>Staph. aureus</i> few colonies	2	5.7
Few colonies; abundant growth <i>Staph. aureus</i>	3	8.6
Few colonies; abundant growth of alpha strep.	3	8.6
Moderate growth; moderate growth of alpha strep.	2	5.7
Numerous; few colonies group G	1	2.8
Group B		
Abundant in pure culture	2	28.6
Predominating abundantly in crypts, few colonies of group B inside	1	14.3
Few colonies, abundant growth of <i>Staph. aureus</i>	2	28.6
Numerous colonies, few colonies of <i>Staph. aureus</i>	1	14.3
Moderate growth, sparse growth of <i>Staph. aureus</i>	1	14.3
Group C		
Abundantly predominating or in pure culture	8	57.1
Abundantly predominating in crypts, few group B colonies inside	1	7.1
Few colonies, <i>Staph. aureus</i> abundantly predominating	3	21.4
Few colonies and Gram negative cocci predominating	2	14.2
Group G		
Abundantly predominating or in pure culture	4	28.4
Moderate growth, <i>Staph. aureus</i> predominating	5	35.7
Predominating, few colonies of <i>Staph. aureus</i>	2	14.2
Moderate growth with moderate growth of <i>Staph. aureus</i>	2	14.2
Few colonies, abundant growth of group A	1	7.1
Group F		
Abundantly predominating	1	33
Numerous colonies mixed with abundant growth of <i>Strep. viridans</i>	1	33
Moderate growth and Gram negative cocci in sparse numbers	1	33

* These figures refer to the percentage distribution of strains of the different groups among the tonsils from which organisms of that particular group were isolated.

tonsils and other sources was attempted utilizing the method described by Swift *et al.*⁴ The results are given in Table III.

Representatives of the following types were found among the strains tested: 44, 41, 1, 14, 19, 33, 4, 36 and 39. Approximately

one-half of the cultures were negative with all the serums tested.[†] About the same proportion of untypable strains was isolated from the different sources. Types 44 and 41 were the ones more commonly encountered. The number of strains studied being too small no

TABLE I.
Fermentation Reactions* of Lancefield's Group "B" Streptococci Obtained from Human and Bovine (Mastitis) Sources.

No. of											Mannitol		
	Bovine strains		Lactose	Dextrose	Saccharose	Maltose	Salicin	Levulose	Trehalose	Glycerol	Dextrin	Galactose	Xylose
	Human strains												Rhamnose
													Adonitol
													Erythritol
													Sorbitol
													Arabinose
													Amigdaline
	9/5		+	+	+	+	+	+	+	+	+	+	—
	1/0		+	+	+	+	+	+	+	—	—	+	—
	1/0		+	+	+	+	—	+	+	—	+	+	—
	18/2		+	+	+	+	+	+	+	—	+	+	—
	1/0		+	+	+	+	±	+	+	—	±	+	—
	1/0		+	+	+	+	—	+	+	—	±	+	—
	1/0		+	+	+	+	±	+	+	—	+	+	—
	3/0		+	+	+	+	±	+	+	—	±	+	—
	3/5		+	+	+	+	+	+	+	±	+	+	—
	7/0		+	+	+	+	+	+	+	±	±	+	—
	9/7		—	+	+	+	+	+	+	+	+	+	—
	0/1		—	+	+	+	+	+	+	±	+	+	—

Total No. of human strains examined—20.

Total No. of bovine strains examined—50

* Observations were made daily during a period of two weeks.

TABLE II.
Fermentation of Glycerol by Lancefield's Group "B" Streptococci of Human and Bovine Origin.

No. and % of strains	Bovine			Human		
	+	±	—	+	±	—
No.	9	15	26	12	6	2
%	18	30	52	60	30	10

+, Positive; ±, Doubtful; —, Negative.
Period of observation—2 weeks.

The fermentation of glycerol is summarized in Table II.

Simmons and Keogh⁴ reported that all their human strains fermented glycerol. They stated, however: "Fermentation of glycerol was not apparent until the third to the sixth day; a few strains did not ferment until later, up to the 14th day." We found also that the fermentation of glycerol was much delayed. The positive strains took from 6 to 13 days to ferment this substance.

All our human strains fermented salicin overnight. Two of the bovine strains failed to ferment this substance and 3 gave a doubtful reaction after 2 weeks.

No strains fermented mannitol, xylose, dulcitol, rhamnose, adonitol, erythritol, sorbitol, arabinose or amigdaline.

Twelve human strains showed the same

fermentation reactions of one or more of the bovine cultures.

None of the bovine strains and only 2 human strains reduced methylene blue in 1:5000 dilution. Twenty bovine strains and all the human strains reduced this dye in 1:20,000 dilution.

All the bovine cultures and 18 of the 20 human cultures hydrolyzed sodium hippurate. No strains were able to split aesculin.

Virulence for mice. Fifty bovine and 20 human strains were tested for virulence. The organisms were grown in 5 cc of tryptose phosphate broth (Difco). The 18-hour growth was used for inoculation. All the mice used came from the same colony and weighed from 16 to 20 g approximately. Two animals were inoculated intraperitoneally with 0.5 cc and 0.1 cc respectively of each culture. After death the heart's blood was cultured and if streptococci were present they were grouped by Lancefield's method¹¹ utilizing Fuller extracts.¹⁰

Only 2 bovine strains (4%) killed mice when 0.5 cc of the culture was given intraperitoneally. In both cases group B streptococci were recovered from the heart's blood.

¹¹ Lancefield, R. C., *J. Exp. Med.*, 1933, 57, 571.

Biological Properties and Mouse Virulence of *Streptococcus agalactiae* and Lancefield's Group "B" Streptococci from Human Sources.*

A. POMALES-LEBRÓN, P. MORALES-OTERO, AND J. BARALT.

From the Department of Bacteriology of the School of Tropical Medicine, San Juan, P.R.

During a bacteriological study of cow mastitis¹ a large number of strains of *Streptococcus agalactiae* (Lancefield's group B) were isolated. In the course of an investigation of the bacteriology of excised tonsils Lancefield's group B streptococci were isolated from 6% of the tonsils examined.² We have occasionally isolated group B streptococci from other pathological conditions. Other workers have isolated group B streptococci from various pathologic processes in humans. The bovine udder has been suspected by some as the ultimate source of group B streptococci from human sources. Careful studies have been made by others^{3,4} in an attempt to prove or disprove this point. Further investigation is necessary to find more evidence that might throw light on this problem.

Fifty strains of *Streptococcus agalactiae* (Lancefield's group B) isolated during the study of cow mastitis and 20 strains from excised tonsils and other human sources have been studied in detail and the results compared.

Methods. Carbohydrate and other media. Beef heart infusion broth (Difco) was used as a basic medium with Brom-cresol purple as indicator. The carbohydrate solutions were sterilized by filtration and then added to 5 cc of the sterile basic medium to give a final concentration of 1%. The sugar media were inoculated with one drop of an 18-hour growth of the streptococci in beef

heart infusion broth. The aesculin medium was prepared according to Diernhofer⁵ as cited by Plastringe *et al.*⁶

The methylene blue milk medium was prepared containing 1:5000 final dilution of the dye as recommended by Avery⁷ and also containing the dye in 1:20,000 dilution. The hydrolysis of sodium hippurate was tested by growing the organisms in the original medium described by Ayers and Rupp⁸ as well as in that recommended by Coffey and Foley.⁹

Grouping of streptococci. We used commercial grouping serums or antisera prepared by us by immunizing rabbits with standard strains supplied by Dr. Lancefield. The extracts for the precipitin tests were made by Fuller's¹⁰ method.

Results. The fermentation reactions of all the human and bovine cultures are given in Table I.

All the bovine organisms fermented lactose. Eight of the 20 human strains did not ferment this substance. All the human and bovine strains fermented dextrose, saccharose, maltose, levulose, trehalose and galactose. The great majority of the cultures fermented these substances overnight. All the human strains fermented dextrin, one bovine strain did not ferment this substance and 10 gave a doubtful reaction.

⁵ Diernhofer, K., *Milch-wirtschaft. Forsch.*, 1932, **13**, 368.

⁶ Plastringe, W. N., Anderson, E. O., Brigham, G. D., and Spaulding, E. H., *Storrs Agric. Exp. Sta. Bull.* 195, 1934.

⁷ Avery, R. C., *J. Exp. Med.*, 1929, **50**, 463.

⁸ Ayers, S. H., and Rupp, P., *J. Infect. Dis.*, 1922, **30**, 388.

⁹ Coffey, J. M., and Foley, G. E., *Am. J. Pub. Health*, 1937, **27**, 927.

¹⁰ Fuller, A. T., *Brit. J. Exp. Path.*, 1933, **19**, 130.

* This work has been possible through a grant of the insular Department of Agriculture.

¹ Pomales-Lebrón, A., Baralt, J., and Morales-Otero, P., in press.

² Pomales-Lebrón, A., Morales-Otero, P., and Baralt, J., in press.

³ Brown, J. H., *J. Bact.*, 1939, **37**, 133.

⁴ Simmons, R. T., and Keogh, E. V., *Australia J. Exp. Biol. and Med. Science*, **18**, 151.

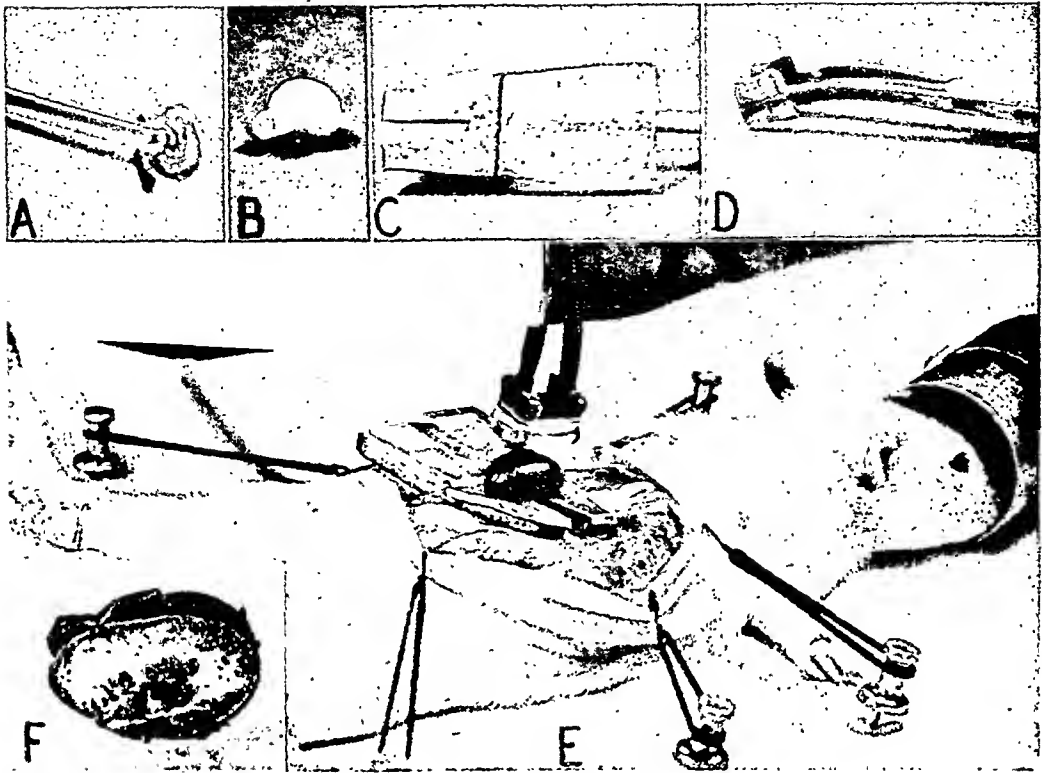


FIG. 1.

- A. Glass mold.
- B. Completed latex rubber capsule removed from mold.
- C. V-shaped platform for stabilizing the delivered kidney while the capsule is being applied.
- D. Kelly hemostat with ends broadened by means of solder to stretch and hold the everted capsule during application to kidney.
- E. Operative field showing kidney in place on platform with stretched latex capsule held over the kidney prior to application.
- F. Sagittal section of kidney showing thick, tough capsule 57 days after latex rubber capsule was applied. Rubber capsule itself has been removed completely.

about 90% of 60-day survivors. The convenience and dependability of the method, particularly when large numbers of rats with stable hypertension are needed, makes it worthy of brief description.

Method. A series of glass molds, resembling rats' kidneys in size and shape, were made by fusing and shaping solid glass rods or tubes (Fig. 1A). The molds were then dipped into liquid latex and placed in a rack, stem downward, to dry. Three dippings, producing a thickness of approximately 0.003 inch, plus a heavier reinforcing coat applied with camel's hair brush in the angle between the capsule and its neck made the rubber membrane suitably rugged and elastic. The

completed capsules, still on the molds, were soaked in running hot tap water (45-50°C) for several hours to toughen the rubber, dissolve residual protein, and assist "peeling-off" the finished capsules (Fig. 1B). Assorted sizes of completed capsules were stored in 75% alcohol until used.

Male rats, weighing 190 to 200 g, were anesthetized with ether and fastened in the prone position (Fig. 1E). The hair over the lumbar region was removed and the skin scrubbed with 75% alcohol. Through a single dorsal mid-line incision, 5 to 6 cm long, either kidney could be delivered by the usual approach, *i.e.* by cutting the subcutaneous fascia longitudinally and splitting the under-

None of the animals receiving 0.1 cc of the culture died during the 10-day period of observation. Thirteen human strains (65%) killed mice when 0.5 cc of culture was injected intraperitoneally.

Of these 13 strains 3 also killed when 0.1 cc was inoculated. All the animals died in from 24 to 48 hours after inoculation.

Summary and conclusions. The biological properties and mouse virulence of 70 Lancefield's group B cultures (50 bovine and 20 human) have been studied and compared. The fermentation reactions show that, aside from the inability to ferment lactose by 8 of the human strains and some difference in the fermentation of glycerol, dextrin and salicin, the reactions are very similar for both groups. In some instances the biological properties studied were identical for strains

of bovine and human origin. It has been observed with the majority of strains that the differences between single bovine and human cultures were not greater than those between individual strains from the same source.

There is an indication that human strains are more likely to reduce methylene blue.

It is known that the mouse virulence of group B streptococci is low. Inoculation of the 70 cultures studied into mice gave results which indicated that the strains from human sources possessed a higher mouse virulence than the bovine strains.

In spite of minor differences between cultures from bovine and human origin, their similarity in other respects points to a close relationship between these organisms.

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Latex Rubber Capsule for Producing Hypertension in Rats by Perinephritis.

MORRIS ABRAMS AND S. SOBIN. (Introduced by E. M. Landis.)

From the Department of Physiology, Harvard Medical School, Boston, Mass.

In studies of dietary choices by hypertensive rats¹ it was necessary to produce large numbers of animals with graded and persistent hypertension. Constricting perinephritis was the method of choice for several reasons but the use of cellophane,^{3,4} collodion⁵ or silk^{5,6} in young rats is technically diffi-

cult, the mortality is high, and the results are variable. Partial nephrectomy² could not be used because of the accompanying trauma and renal insufficiency. Simple compression of the renal artery had the disadvantage of technical difficulty coupled with later development of collateral circulation.

Enveloping the kidneys of young rats in molded pure gum latex capsules had previously been used to produce hypertension in a few animals in this laboratory.⁷ This paper describes more complete experiments, indicating that this method can produce persistent hypertension ranging from 165 to 240 mm Hg at the end of 2 or 3 weeks in at least 70% of the total operated rats and in

¹ Abrams, M., and Landis, E. M., to be published.

² Chanutin, A., and Ferris, E. B., Jr., *Arch. Int. Med.*, 1932, **49**, 767.

³ Friedman, B., Jarman, J., and Klemperer, P., *Am. J. Med. Sci.*, 1941, **202**, 20.

⁴ Schroeder, H., *J. Exp. Med.*, 1942, **75**, 513.

⁵ Grollman, A., and Williams, J. R., Jr., *Am. J. Med. Sci.*, 1942, **204**, 73.

⁶ Kempf, G. F., and Page, I. H., *J. Lab. and Clin. Med.*, 1942, **27**, 1192.

⁷ Sobin, S., *Am. J. Physiol.*, 1946, **140**, 179.

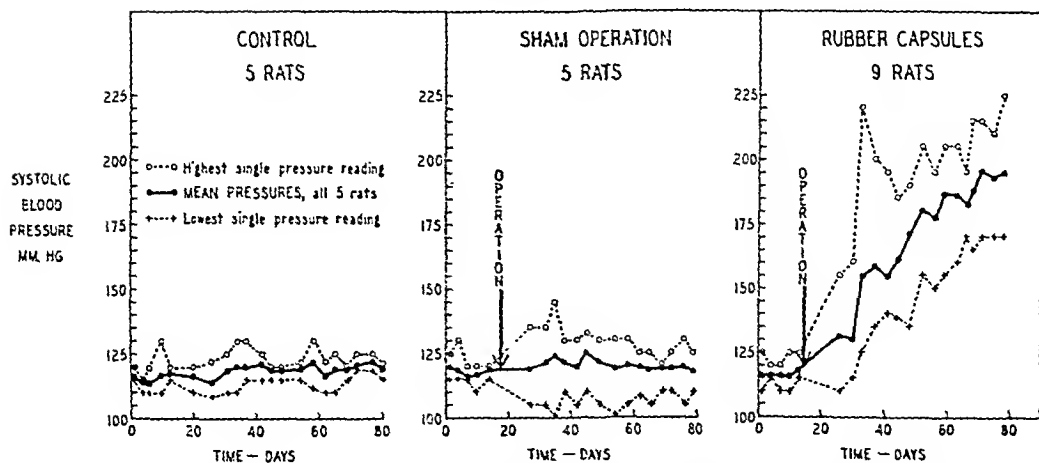


FIG. 2.

Charts showing systolic blood pressures of control rats, of sham-operated rats, and of rats with perinephritic hypertension due to bilateral application of rubber capsules.

TABLE II.

Comparison of Blood Pressures Measured (a) plethysmographically and (b) by Hamilton manometer.

	Indirect readings (plethysmograph on tail) Systolic mm Hg	Direct readings (Hamilton manometer, femoral artery)	
		Systolic mm Hg	Diastolic mm Hg
Control rats	125-130	130-140	80- 85
	125-130	125-140	85- 90
	115-120	115-125	80- 90
Sham-operated rats (60+ days post-op.)	122-127	120-130	90- 95
	105-110	100-115	60- 70
	125-130	130-140	80- 90
Rats with bilateral latex rubber capsules (60+ days post-op.)	185-190	190-210	100-120
	205-210	190-220	110-130
	200-205	200-210	140-150

165 mm Hg or more was regarded as significant, *i.e.* any animal showing a rise less than 35 mm Hg above the highest single reading ever observed in parallel control animals was recorded as a failure.

The results for a total of 47 operated rats have been combined in Table I. As might be expected, experience led to improved results in the second series. In the first group mortality was 30%, in the second 10%. Similarly the incidence of significant hypertension was greater in the second and technically superior series. Severe hypertension (systolic blood pressures between 185 and 240 mm Hg) developed in 70% of the second

series; moderate hypertension (165 to 185 mm Hg) in 20%.

Fig. 2 compares, for the second series, the highest single blood pressure, the lowest single blood pressure, and mean pressures for (a) 5 control rats, (b) 5 sham-operated rats, and (c) the 9 successfully operated rats all of which developed significant hypertension. The sham operation produced only a transitory increase in the systolic pressure of a few animals, while mean pressure for the group was not modified. Applying the latex capsules led to a clear increase in mean pressure by 2 to 3 weeks after operation and all of the animals were clearly developing hy-

TABLE I.
Systolic Blood Pressures (Indirect) 60 Days After Applying Latex Rubber Capsules Bilaterally.

	First series	Second series	Totals, both series	%, both series together
Total No. operated	37	10	47	100
Deaths—1 to 30 days				
1 to 5 days post-op.	7	0	7	
6 to 30 days post-op.	4*	1	5	25.5
Survivors—30 days or more				
Systolic blood pressures†				
185 to 240 mm Hg	13‡	7	20	42.5
165 to 185 mm Hg	10§	2	12	25.5
135 to 165 (failures)	3	0	3	6.5

* Sacrificed 30 days after operation because of infected wounds and/or emaciation.

† Concurrent control rats, 11 in all, had systolic blood pressures ranging from 100 to 130 mm Hg.

‡ One died 57 days after operation in severe cardiac failure.

§ One died 42 days after operation with gross hematuria and acute urinary retention.

lying dorsal muscle mass by blunt dissection. In addition, splitting the muscles close to the vertebral column avoided undue stretching of the renal vessels during further manipulation of the kidney.

The upper and lower poles of the kidney were freed gently from the adrenal glands and perirenal fat by blunt dissection. Decapsulating the kidneys decreased the immediate postoperative mortality from renal insufficiency and was accomplished by nicking the capsule and peeling it toward the hilus with tissue forceps. Any oozing of blood was readily controlled by gentle pressure with a moist sponge.

The liberated and decapsulated kidney was then placed on the platform (Fig. 1C and E) with its pedicle in the V-shaped cleft. The soft rubber covering of this platform reduced trauma and prevented slippage. A rubber capsule of a size which would snugly enclose, but not compress, the kidney was rinsed free of all alcohol in warm Ringer's solution and placed in the everted position over the widened end of the hemostat used as an applicator (Fig. 1D and E). With the handles of the hemostat spread, the widely expanded rubber capsule was brought down over the kidney and the ends of the applicator pushed firmly against the kidney platform.

The lower free border of the everted capsule was drawn over the lower pole of the kidney by means of forceps, after which the

other end of the capsule applicator was quickly turned inward, causing the upper end of the capsule, guided by forceps, to snap in place over the upper pole of the kidney. The loose neck of the capsule did not compress the renal vessels or ureter. The first kidney having been replaced, the other was treated similarly at the same operation and the rats were placed in individual metabolism cages for recovery. If oliguria or gross hematuria appeared during the night, 5 cc of Ringer's solution were given intraperitoneally and repeated again in 12 hours.

Sham operations, including all steps except the final snapping on of the latex capsule were performed in 5 animals. Systolic blood pressures in control, sham-operated and experimental rats were measured indirectly twice weekly by the plethysmographic technic described by Sobin.⁷ All rats received Purina laboratory chow and water *ad lib*.

Results. Table I summarizes results obtained 60 days after bilateral application of latex rubber capsules in (a) an exploratory series of 37 male rats and (b) a more completely studied and technically superior series of 10 male rats. The highest normal systolic blood pressure in a parallel control series of 11 male rats was 130 mm Hg, the lowest 100 mm Hg. Because small changes in blood pressure are apt to be misleading, a rise of systolic blood pressure to

TABLE I.
Comparison of Urea- and Phenol-treated Skin Test Antigens.

Susp. 96 10% saline Susp.-Y's membranes fractions:	Description	Complement fixation filter with standard positive serum pool	Skin reactions with antigens diluted:			
			1-500*		1-2000†	
			Pt. weak reactors†	7 days	Pt. moderate reactors†	7 days
A	Inactivation 3 wks with: Urea 2% 2-4°C	Preservative added to diluted antigens Merthiolate 0.25% 1-20,000	Imm	30†	Dea	DeL
			Bla	36	den	Ben
			Wll	4	DeV	Tho
B	Urea 2% 2-4°C	"	Imm	24	Dea	DeL
			Bla	25	den	Ben
			Wll	9	DeV	Tho
C	Phenol 0.5% 37°C	"	Imm	36	Dea	DeL
			Bla	49	den	Ben
			Wll	9	DeV	Tho

* Dilutions are expressed in terms of original yolk sac weight.

† Score equals product of the axes of the erythematous papule in mm.

† Patients used in testing the three dilutions were selected on the basis of previous tests.

ly infected with the virus of *Lymphogranuloma venereum* and fractions thereof were treated as shown in Table I. Fraction A was prepared by the method in use in these laboratories for the routine production of *Lymphogranuloma venereum* skin testing antigens. Fraction B was prepared in identical manner. Fraction C was treated at 37°C for 3 weeks with phenol in a final concentration of 0.5%. This procedure effected enhancement of complement-fixing activity.† The final dilutions of Fractions A and C contained 0.5% phenol and 1-20,000 merthiolate as preservatives, while Fraction B contained only merthiolate. Control material for each fraction was prepared in identical manner from normal yolk sac membranes (in approximately the same stage of development) for simultaneous testing.

When all preparations had passed the necessary tests for sterility and inactivation of virus, they were tested on clinically and serologically positive cases of *Lymphogranuloma venereum*, using the 1-500 dilutions of each preparation on each of several patients, the 1-2000 dilutions on each of another group of patients and the 1-8000 dilutions on each of a third group. Such a procedure was followed to rule out differences in individual response to the same test material. The test dose of each preparation was 0.1 ml injected intracutaneously on the flexor surface of the forearm. The reactions were recorded at 2- and 7-day intervals following injection.

Results. Some patients show, during the first 2 or 3 days following the test injection, a more or less extensive edematous, erythematous area with or without induration. The erythema may recede fairly rapidly, leaving a characteristic erythematous papule which regresses slowly with or without central necrosis, requiring several weeks for its complete disappearance. This more persistent reaction, as seen one week after injection, was thought to present a more reliable criterion for the comparative evaluation of the various preparations. Table I, therefore, shows only the 7-day readings. The product of the axes of the erythematous papule in mm was used as a basis for scoring

pertension by 3 weeks after operation.

Systolic blood pressures having been followed by repeated indirect measurements for 60 days, these conclusions as to the severity of the hypertension were verified by direct determinations using the Hamilton manometer. Three rats of each group were lightly anesthetized by injecting intraperitoneally 3 mg sodium pentobarbital per 100 g body weight. Their pressures were measured (a) plethysmographically and (b) immediately afterward directly from the femoral artery. Table II summarizes this comparison and, within the usual respiratory variations of blood pressure when a tracheal cannula is not used, shows the quantitative accuracy of conclusions drawn from previous indirect measurements.

Comment. The pathogenesis of hypertension in these animals appears to be similar

to that produced by other chronically irritative materials such as silk or cellophane. Within a few days after the application of the rubber capsule the superficial tissues of the renal cortex reacted by forming first a fibrinous, then a definitely fibrous, envelope. The latter can become very thick and tough as shown in Fig. 1F. In this specimen the latex capsule has been applied to the kidney 57 days before. The microscopic changes were also similar to those produced by other types of irritative capsule. Similar results have also been observed in a few rabbits.

Summary. Enclosing both kidneys in molded latex rubber capsules to produce perinephritis can be recommended for its simplicity and effectiveness, particularly when large numbers of hypertensive rats are required.

15812

On Use of Urea- and Phenol-Treated Skin Test Antigens for Diagnosis of *Lymphogranuloma venereum*.

CLARA NIGG, A. W. GRACE, AND MAURICE R. HILLEMANN.

From the Virus Laboratories, E. R. Squibb and Sons, New Brunswick, N.J., and Long Island College Hospital, Brooklyn, N.Y.

It has been shown in an earlier report from this laboratory¹ that urea in concentrations of 1 to 2% inactivated the virus of *Lymphogranuloma venereum* in 10% yolk sac suspensions prepared from infected chick embryos. Suspensions so inactivated with urea were satisfactory both as complement-fixing and skin test antigens. Urea-inactivated skin test antigens (containing 0.25% phenol and 1-20,000 merthiolate as preservatives) have been prepared in these laboratories for clinical testing for over 4 years.²⁻⁵

When later studies^{6,7} revealed that the addition of phenol to yolk sac suspensions enhanced their complement-fixing activity from a titer of approximately 1-200 to 1-3200, and in some instances to 1-6400, the question of the possible enhancement with phenol of the skin test activity naturally presented itself.

Methods. To investigate the effect of phenol on the reactivity of skin test antigens, saline suspensions were prepared as previously described⁷ from yolk sac membranes heavily

¹ Nigg, C., *Proc. Soc. Exp. Biol. and Med.*, 1942, 49, 132.

² Grace, A. W., Rake, G., and Shaffer, M. F., *Proc. Soc. Exp. Biol. and Med.*, 1940, 45, 259.

³ Blair, J. E., *J. Immunol.*, 1944, 49, 63.

⁴ Florman, A. L., *J. Immunol.*, 1945, 51, 29.

⁵ Dulaeay, A. D., and Packer, H., *J. Immunol.*, 1947, 55, 53.

⁶ Nigg, C., and Bowser, B., *Proc. Soc. Exp. Biol. and Med.*, 1943, 53, 192.

⁷ Nigg, C., Hilleman, M. R., and Bowser, B., *J. Immunol.*, 1946, 53, 259.

infection of the mouse with a single intraperitoneal dose of 0.02 g/kg, while the maximum tolerated dose, intraperitoneally, was found to be 0.12 g/kg, corresponding to a therapeutic index of 6. The immediate spirochetocidal effect of (II) was screened in rabbit syphilis: 16 animals with fully developed, dark-field positive chancres were treated with a single subcutaneous injection of (II) dissolved in propylene glycol in doses ranging from 2.5-40 mg (.0008-.013 g/kg). In all animals treated with doses equal to or greater than .006/kg the spirochetes were not demonstrable 5 days after the treatment. As to toxicity, a single intramuscular dose of .05 g/kg is well tolerated while .08 g/kg is lethal.

It follows from these experiments that the condensation product of a chemotherapeutically active arsenical with BAL may result in a new compound combining relatively low toxicity with a significant trypanocidal and spirochetocidal activity.

Similar results, to be reported in a forthcoming publication, have been obtained with analogous BAL derivatives of other aromatic arsenicals as well as with aromatic antimony compounds, including arsanilic acid, tryparsamide, carbarsone, stibanilic and acyl stibanilic

acids.

Hence, in a general manner, inclusion of a phenyl substituted arsenic or antimony residue in a 5-membered sulfur-containing ring as in (II) is not incompatible with a significant chemotherapeutic activity.

The new BAL compounds seem of more than theoretical interest in view of their stability and solubility properties. As a group they are more soluble in nonaqueous solvents than the parent organometallics, a fact which may well have repercussions on their chemotherapeutic spectrum. The solubility, and consequently, the distribution within the organism, may readily be modified by replacing the BAL radical in the above-mentioned compounds by other dithiols, such as 2,3-dimercaptopropionic acid, BAL ethers, BAL glucoside, 1,2-dimercaptobenzene, etc.

The mechanism of the therapeutic effect of the BAL derivatives remains to be elucidated. There are at least 2 possibilities: (1) the molecule may act as a whole, which would imply a mechanism not foreseen by the "Ehrlich-Voegtlin SH-arsenoreceptor theory," (2) the BAL compounds are dissociated in the organism and act essentially like the parent compounds.

15814

Epithelial Keratinization as Evidence of Fetal Vitamin A Deficiency.*

JAMES G. WILSON AND JOSEF WARKANY.

From the Department of Anatomy, School of Medicine and Dentistry, University of Rochester and from the Children's Hospital Research Foundation, Department of Pediatrics, College of Medicine, University of Cincinnati.

The appearance of stratified keratinized epithelium in places where such epithelium is not ordinarily found is the cardinal sign of vitamin A deficiency. It has been stated that this histologic change, referred to as keratinizing metaplasia, occurs in all verte-

brates regardless of age.¹ However, it has not been described in fetal or newborn animals whose mothers were in a state of vitamin A deficiency during pregnancy. This is noteworthy, since Hale,² using the pig, and Warkany and Schraffenberger,³ using the rat, obtained fetuses that bore severe morphologic

* This work was aided in part by a grant from the Nutrition Foundation, Inc., N. Y.

¹ Wolbach, S. B., and Bessey, O. A., *Physiol. Rev.*, 1942, **22**, 233.

² Hale, F., *Am. J. Ophth.*, 1935, **18**, 1087.

³ Warkany, J., and Schraffenberger, E., *Arch. Ophth.*, 1946, **35**, 150.

the reactions. In no case did the control Fractions A, B and C, give a positive reaction with 1-500 dilution. The results present at the most only slight and probably insignificant differences in reactivity between the 3 preparations, whether phenol was used in the preparations (Fractions A and C) or was completely absent (Fraction B).

The skin test results are in contrast to the comparative complement-fixing activities of Fractions A and B with a titer of only 1-200, and Fraction C, with a titer of 1-3200,

constituting a 16-fold enhancement.

Summary. 1. Saline suspensions prepared from yolk sacs heavily infected with the virus of *Lymphogranuloma venereum* and inactivated with 2% urea or 0.5% phenol constituted satisfactory diagnostic skin test antigens in high dilutions.

2. Yolk sac suspensions treated with phenol so as to enhance their complement-fixing activity 16-fold were not appreciably more active as skin test antigens than urea-treated suspensions.

15813 P

Trypanocidal and Spirochetocidal Compounds Derived from BAL and Organic Arsenicals.

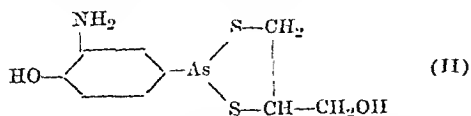
ERNST A. H. FRIEDHEIM AND HENRY J. VOGEL. (Introduced by M. B. Sulzberger.)

From the Laboratory of E. A. H. Friedheim, New York City.

This study is part of an investigation of the chemotherapeutic activity of organo-metallic compounds as affected by chemical combination with dithiols.

War research has brought out the fact that BAL (British Anti Lewisite, 2,3-dimercaptopropanol) protects man and experimental animals against the toxic effect of organic arsenicals, including 4-arsenoso-2-aminophenol (I), *i.e.*, oxophenarsine U.S.P., and counteracts *in vitro* the trypanocidal effect of this drug.¹⁻³

The limited information published to date (February 18, 1947) suggests that the compound resulting from the condensation of I with BAL, *i.e.*, 2-amino-4-[methylol-(ethylenedimercaptoarsino)]-phenol (II)



would be significantly less toxic than its parent substance (I), but devoid of chemotherapeutic activity.

With a view to evaluating this proposition, we have synthesized (II) and tested *in vivo* its trypanocidal and spirochetocidal activity.

(II) crystallizes from methanol as white needles, soluble in propylene glycol and dilute hydrochloric acid, insoluble in water and anhydrous acetone. It gives a negative nitroprusside reaction at pH 8, positive at 10.

Analysis:

Calcd. for $C_9H_{12}O_2NS_2As$:	N 4.59, As 24.6
Found:	N 4.52, As 24.0
	4.49 24.8

(II) forms a crystalline hydrochloride (III), soluble in water, ethanol and propylene glycol, insoluble in anhydrous acetone.

Analysis:

Calcd. for $C_9H_{13}O_2NClS_2As$:	N 4.11; As 22.0
Found:	N 4.10; As 21.2

The free base (II), and more so its hydrochloride (III), are quite stable. Propylene glycol solutions of the hydrochloride may be sterilized by heating for one hour at 100°.

(III) cures the experimental *T. equiperdum*

¹Peters, R. A., *Nature*, November 24, 1945.

²Waters, L. A., and Stock, C., *Science*, 1945, **102**, 601.

³Sulzberger, M. B., and Baer, R. L., *J. Am. Med. Assn.*, 1947, **133**, 293.

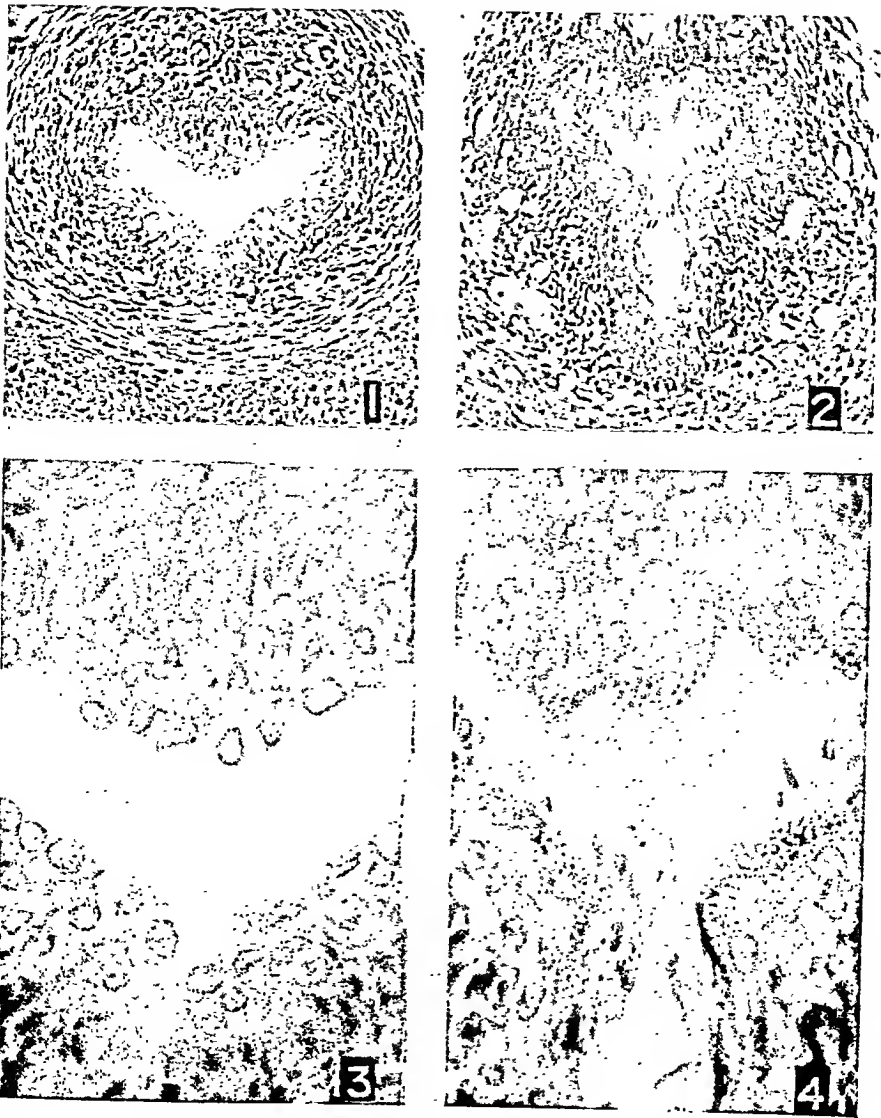


Fig. 1-4.

Fig. 1. Urethra at the level of the urethral sinus of a newborn rat, offspring of a mother fed an adequate diet. ($\times 210$)

Fig. 2. Urethra at the level of the urethral sinus of a newborn rat, offspring of a mother fed a vitamin A-deficient diet. ($\times 210$)

Fig. 3. Higher magnification of the urethra shown in Fig. 1. ($\times 650$)

Fig. 4. Higher magnification of the urethra shown in Fig. 2. ($\times 650$)

These photomicrographs are oriented with the "dorsal" wall toward the top of the page. As used here, the term "dorsal" refers to that wall of the urethra derived from the dorsal wall of the embryonic urogenital sinus, and does not accurately describe the relative position of this wall in the newborn rat.

surface-cells, were observed in the region of the upper sinus that roughly corresponded to the neck of the bladder.

Fetuses taken on the 21st and newborns

taken on the 22nd day showed perhaps a more advanced degree of cornification (Fig. 1-4), with considerable desquamation at some points, but the extent of the affected area was

anomalies when the mothers were deprived of vitamin A during pregnancy.

The present communication is a report of the finding of keratinizing metaplasia in the genito-urinary tract of fetal rats taken from mothers that were deficient in vitamin A during pregnancy. It is based on a study of serial sections of 14 fetuses and newborns from as many different litters, the gestational age of which ranged from the 16th through the 22nd day. Maternal deficiency was induced and maintained by the same diets and experimental procedures as were used by Warkany and Schraffenberger.³ Fetuses of comparable ages and newborn rats from mothers fed an adequate diet were sectioned and studied as controls in order to rule out the possibility of keratinization of genito-urinary epithelia by maternal estrogens during the latter part of pregnancy. The histology of the normal epithelia will not be described in this paper, and only those histologic features which represent distinct deviations from the normal will be described for the A-deficient animals. Nevertheless, it should be stated that keratinization normally does not occur in any part of the genito-urinary tract of fetal and newborn rats except in the most distal portion of the urethra, *i.e.*, the external orifice and a short segment contiguous with it.

Neither the control fetuses nor those subjected to maternal vitamin A deficiency exhibited keratinization in any part of the body prior to the 18th day of gestation. All fetuses older than 18 days whose mothers were fed the A-deficient diet, however, showed keratinizing metaplasia in at least some of the genito-urinary epithelia which constituted, or were derived from, the urogenital sinus. A precise localization of the affected epithelia in terms of definitive organs is not feasible in the present report, since the differentiation of the entire urogenital sinus was conspicuously retarded and this retardation, in turn, complicated by the occurrence of several frank malformations. Until a larger series of animals has been studied and plastic reconstructions prepared, no attempt will be made to analyze the malformations, and the

location of areas of metaplasia will be described only in a general way.

The 18th day of gestation was the earliest time at which metaplastic keratinization appeared in the offspring of A-deficient mothers. A male fetus of this age was found to possess a small area of truly keratinized epithelium within the urethral plate and another area in the pelvic part of the urogenital sinus where, although no completely keratinized cells were seen, the cytoplasm of superficial cells was hyalinized and contained keratohyalin granules. These changes might be designated "prekeratinization." The findings seem particularly interesting in view of the fact that the epidermis and other ultimately cornified epithelia had not undergone keratinization in either this animal or control fetuses of the same age.

True keratinization, typified by cornification and desquamation of the surface-cells and by hyalinization and keratohyalin granulation in the superficial layers of cells, was consistently found in fetuses of a gestational age of 20 days. It was limited, however, to the distal two-thirds of the urogenital sinus, specifically to the segment lying between the point of juncture of the genital ducts with the sinus, cephalically, and the exterior of the body, caudally. Metaplasia occurred as a continuous strip along the dorsal wall of this segment but, curiously, had affected to a much less striking degree, or not at all, the lateral and ventral walls of this region of the sinus. The point at which keratinization was most pronounced, both in degree and in circumferential extent, was where the genital ducts joined the sinus. This was also the most cephalic part of the affected area. This fact speaks against the possibility that keratinizing changes spread into and along the sinus from the already keratinized epidermis on the exterior, and seems to indicate instead that an *in situ* transformation to a keratinized type of epithelium had occurred. No true cornification was seen in 20-day fetuses above the point of entry of the genital ducts, but prekeratinization changes, beginning hyalinization of the cytoplasm and the appearance of keratohyalin granules in the

TABLE I.

Immunity in Mice Given Living and Killed *Shigella sonnei* by Gavage. Challenged Intraperitoneally with 0.5 to 1280 MLD of Homologous Culture.

Type of antigen	% of mice surviving No. of doses of antigen					
	3	4	5	6	7	8
Living culture	57*	55	72	89	73	92
Killed "	70	50	71	76	85	83

* Each % is based on results with 30 mice.

TABLE II.

Immunity in Mice Drinking Killed *Shigella sonnei* in Place of Water for 21 Days.

No. of mice	Fluid consumed	Homologous challenge intraper. MLD	No. of mice surviving
36	Killed culture*	2 to 2048	36
30	Sterile broth	"	2
31	Water	"	2
36	"†	"	0

* 7.5 billion per day per mouse.

† Virulence control.

160,000 MLD of homologous culture suspended in sterile mucin.¹ All mice survived except one which received 160 MLD.

Smaller numbers of living or heat-killed bacteria were compared for their ability to stimulate active immunity when administered by gavage. Six groups of 30 mice were given 3 to 8 daily doses of 1.4 billion living *Shigella sonnei*. Six other groups of 30 mice were given the same number of doses of 1.1 billion killed organisms. Seven days after the last doses each group of 30 mice was divided into 6 equal groups and challenged intraperitoneally with 0.5 to 1280 MLD of homologous culture suspended in mucin. The percentages of mice surviving are found in Table I. The striking finding was that both living and

killed culture yielded similar immunity which increased with increasing number of doses.

The majority of mice remained well during the time they were receiving living culture intragastrically. During this period and for 24 to 48 hours subsequently stool cultures were positive.

II. *Voluntary Drinking of Killed Culture in Place of Water.* A group of 36 white mice were given killed broth culture of *Shigella sonnei* in their water bottles in place of water over a period of 21 days. Another group of 30 mice were given sterile broth in place of water for the same length of time. A third group of 31 mice received only water to drink. Four days after the 21-day period each group of mice was challenged intraperitoneally with 2 to 2084 MLD of homologous culture in mucin. Data in Table II show that all mice survived which had ingested killed culture in place of water; that only 2 mice survived which had consumed sterile broth; and only 2 mice survived which had consumed water. The quantity of killed culture, sterile broth and water consumed daily by each mouse was very similar.

Conclusions. Six daily doses of 49 billion living *Shigella sonnei* administered by gavage to white mice stimulated complete immunity to intraperitoneal injection of 16 to 160,000 MLD of homologous culture suspended in sterile mucin. Killed bacteria in doses of 1.1 billion and living bacteria in doses of 1.4 billion stimulated immunity, the titer of which increased with the number of daily doses. There was no significant difference in the immunizing effects of living and killed culture. Mice which drank killed broth culture in place of water for 21 days were immune against 2 to 2048 MLD of homologous culture injected intraperitoneally.

¹ Cooper, Merlin L., and Keller, Helen M., *J. Pediat.*, 1941, 18, 451.

scarcely greater than at 20 days. Again, structures derived from the urogenital sinus distal to its juncture with the genital ducts were keratinized throughout their length; and metaplasia was always more pronounced on, and tended to be limited to, the dorsal aspect of these structures. In one newborn male an area of true metaplasia was observed in the vesico-urethral portion of the sinus at a point corresponding to the upper prostatic urethra. Otherwise, only pre-keratinization changes were seen in this more cephalic portion of the sinus. Even in these newborns and more mature fetuses no histologic deviations from normal were seen in the epithelia in regions of the definitive bladder, ureters, kidneys, mesonephric ducts or Müllerian ducts.

Comment. These observations reveal that genito-urinary epithelia in fetal rats, like many other epithelia in older animals, require vitamin A for their morphologic integrity. It appears that this requirement is not manifested by keratinizing metaplasia until the fetal tissues have undergone a certain degree of differentiation. In the urogenital sinus this is attained by, or shortly before, the 18th day of gestation. The fact that keratinization did not affect other epithelia, which in older animals do respond to A-deficiency by undergoing metaplastic

changes, does not prove that the unaffected epithelia were differentiated to a lesser degree than those of the urogenital sinus. Nevertheless, it is suggestive in this direction, and it undoubtedly reflects some unobserved morphologic or perhaps functional difference between those tissues that undergo metaplasia during the fetal period and those that react in this fashion to A-deficiency only at an older age.

The prevalence of congenital malformations in the genito-urinary tract of these fetuses is probably somehow related to the particular susceptibility of epithelia in this region to keratinizing metaplasia, although any attempt to evaluate such a relationship must await a more thorough study of the malformations.

Summary. Keratinizing metaplasia in epithelia of the genito-urinary tract was observed in fetal rats taken from mothers fed a vitamin A-deficient ration. Only structures derived from the fetal urogenital sinus were affected. The earliest metaplastic changes were seen in a fetus of 18 days gestational age; by the 20th day keratinization was seen in the dorsal wall of all organs distal to the termination of the genital ducts. Epithelia in other parts of the body were not affected by these changes.

15815 P

Immunization of Mice with Dysentery Antigen Administered by Gavage and by Voluntary Drinking.*

MERLIN L. COOPER AND HELEN M. KELLER.

From the Children's Hospital Research Foundation, and the Department of Pediatrics, College of Medicine, University of Cincinnati.

I. Living and Killed Culture by Gavage. In the course of attempts to infect white mice by gavage with living *Shigella sonnei*, we have observed the development of immunity. A high degree of immunity follows administration of large numbers of bacteria. Less

immunity follows administration of fewer bacteria.

Fifteen mice were given 6 consecutive daily doses of 49 billion living bacteria with a syringe and blunt needle introduced into the stomach by way of the esophagus. Five days after the last dose, small groups of these mice were challenged intraperitoneally with 16 to

* This study was conducted under a grant-in-aid from the U. S. Public Health Service.

The presence or absence of the adrenal glands modifies the survival of the eviscerated and the eviscerated-nephrectomized rat either with or without insulin. This effect of adrenalectomy can be observed within 2 hours following operation. The data do not permit a differentiation between the relative importance of the adrenal medulla and the adrenal cortex in effecting the survival of the

eviscerated rat.

Summary. The survival of the eviscerated rat is shortened by either nephrectomy or insulin. Insulin shortens the survival of the eviscerated-nephrectomized rat. Adrenalectomy at the time of evisceration shortens the survival of the eviscerated and eviscerated-nephrectomized rat either with or without insulin.

15817

Enhancement and Depression of Neuromuscular Transmission by Intra-arterial Injection of Potassium Chloride in the Dog.

SHEPPARD M. WALKER. (Introduced by A. S. Gilson, Jr.)

From the Department of Physiology, Washington University School of Medicine, St. Louis, Mo.

Rosenblueth and Cannon¹ observed that intra-arterial injection of KCl, during indirect stimulation of muscle in the cat at a high frequency, produced marked depression of muscle tension. More recent studies^{2,3} have shown that intraperitoneal injection of KCl improved neuromuscular transmission in the rat and that intra-arterial injection of KCl in the nerve-muscle preparation from the frog first augmented and then depressed neuromuscular transmission. The present paper is concerned with the effect of varied rates of intra-arterial injection of KCl on the development and maintenance of muscle tension during stimulation of the nerve at a high frequency. Nine dogs anesthetized with sodium barbital were used in the experiments. Both *Triceps surae* were prepared as previously described for the rat.² Spring levels were used for the recording of muscle tension. The muscles of each leg were stimulated simultaneously through the cut sciatic nerves with electrodes connected in parallel and led from a Thyatron stimulator. The stimulus strength was about 6 times threshold

and the frequency of stimulation was 275 to 300 shocks per second. The duration of stimulation was usually 5 or 10 seconds with an equal interval of rest between periods of stimulation. In some experiments stimulation was continuous during the period of injection. In a few experiments the muscles were stimulated with single shocks during the period of injection. Injections were made into the femoral artery. Two rates of injection were employed: slow, 2 to 3 mg of KCl (1.12% solution) per second; fast, 15 mg of KCl (4.2% solution) per second.

Results. During indirect stimulation at 300 shocks per second the peak of tetanic tension was followed immediately by a rapid decline of tension to a much lower level which was either maintained or slightly increased during continued stimulation (Fig. 1, 1A). Slow injection of KCl gradually reduced the rate and the extent of decline of tension in the perfused muscles during successive periods of stimulation. The maximal enhancement of tetanic tension was attained approximately one minute after the beginning of injection (Fig. 1, 1B). In the contralateral muscles, not subjected to KCl perfusion, little change in the tension curve occurred during the same interval (cf. 2A and 2B of Fig. 1). By the end of 2 minutes of slow injection

¹ Rosenblueth, A., and Cannon, W. B., *Am. J. Physiol.*, 1940, **130**, 205.

² Walker, S. M., *Am. J. Physiol.*, 1947, **149**, 7.

³ Walker, S. M., and Laporte, Y., *J. Neurophysiol.*, 1947, **10**, 79.

Effect of Adrenalectomy upon Survival Time of the Eviscerated Rat.

DWIGHT J. INGLE AND JAMES E. NEZAMIS.

From the Research Laboratories, The Upjohn Company, Kalamazoo, Mich.

This is one of a series of studies of endocrine deficiency and excess in the eviscerated rat. It was shown that removal of the adrenal glands shortens the survival of the eviscerated rat under several experimental conditions.

Methods. Male rats of the Sprague-Dawley strain were fed Purina Dog Chow. At a weight of 185 to 205 g, the inferior vena cava was ligated between the liver and kidneys to cause the development of a collateral circulation. Asepsis was preserved. When the animals reached 250 ± 2 g they were anesthetized (intraperitoneal injection of 18 mg of cyclopentenyl-allyl-barbituric acid sodium) and eviscerated.¹ The vascular supply of the adrenals and the kidneys was ligated and these organs removed at the time of evisceration.

The temperature of the room was 75° to 78°F and the humidity 30 to 35% of saturation. Crystalline zinc insulin (Lilly) was given to some of the animals (Exp. 3 and 4). It was injected into the saphenous vein of the right hind leg with an initial dose of 4 units and a subsequent steady rate of 40 units per 24 hours by means of a continuous injection apparatus. Injections were started within 2 minutes following removal of the liver. Cessation of heart beat was the criterion used to judge the time of death.

Experiments and Results. Ten adrenalectomized and 10 nonadrenalectomized rats were studied in each experiment. Comparisons of adrenalectomized and nonadrenalectomized animals were always made simultaneously on pairs.

In Exp. 1 (kidneys intact, no insulin) the nonadrenalectomized animals survived an average of 870 minutes with a range of 535

to 1467, and the adrenalectomized an average of 361 minutes with a range of 125 to 724 minutes.

In Exp. 2 (nephrectomized, no insulin) the nonadrenalectomized animals survived an average of 183 minutes with a range of 152 to 222, and the adrenalectomized an average of 139 minutes with a range of 67 to 175.

In Exp. 3 (kidneys intact, insulin) the nonadrenalectomized rats survived an average of 86 minutes with a range of 65 to 123, and the adrenalectomized an average of 72 minutes with a range of 57 to 99.

In Exp. 4 (nephrectomized, insulin) the nonadrenalectomized rats had an average survival of 76 minutes with a range of 64 to 85, and the adrenalectomized an average of 61 minutes with a range of 43 to 70 minutes.

In 39 of the 40 pairs of rats studied in these experiments the adrenalectomized animal survived a shorter period than its nonadrenalectomized control.

Discussion. The study of the effect of hormone deficiency and excess upon short survival times may, as in the present experiment, provide one index of the time required for hormonal effect.

It was previously known that either nephrectomy² or the administration of insulin³ hastens the onset of hypoglycemia and death of the eviscerated rat. In these experiments it was found that the survival time of the eviscerated-nephrectomized rat is decreased still further by the injection of insulin. All of the animals dying under these conditions show marked hypoglycemia, and die with convulsions and respiratory failure. In each case the symptoms can be prevented and survival greatly prolonged by the administration of glucose.

² Reinecke, R. M., *Am. J. Physiol.*, 1942, **130**, 167.

³ Ingle, D. J., and Sheppard, R., *Endocrinology*, 1945, **37**, 377.

¹ Ingle, D. J., and Griffith, J. Q., Chapter 16, *The Rat in Laboratory Investigation*, J. B. Lippincott Co., Philadelphia, 1942.

The presence or absence of the adrenal glands modifies the survival of the eviscerated and the eviscerated-nephrectomized rat either with or without insulin. This effect of adrenalectomy can be observed within 2 hours following operation. The data do not permit a differentiation between the relative importance of the adrenal medulla and the adrenal cortex in effecting the survival of the

eviscerated rat.

Summary. The survival of the eviscerated rat is shortened by either nephrectomy or insulin. Insulin shortens the survival of the eviscerated-nephrectomized rat. Adrenalectomy at the time of evisceration shortens the survival of the eviscerated and eviscerated-nephrectomized rat either with or without insulin.

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Enhancement and Depression of Neuromuscular Transmission by Intra-arterial Injection of Potassium Chloride in the Dog.

SHEPPARD M. WALKER. (Introduced by A. S. Gilson, Jr.)

From the Department of Physiology, Washington University School of Medicine, St. Louis, Mo.

Rosenblueth and Cannon¹ observed that intra-arterial injection of KCl, during indirect stimulation of muscle in the cat at a high frequency, produced marked depression of muscle tension. More recent studies^{2,3} have shown that intraperitoneal injection of KCl improved neuromuscular transmission in the rat and that intra-arterial injection of KCl in the nerve-muscle preparation from the frog first augmented and then depressed neuromuscular transmission. The present paper is concerned with the effect of varied rates of intra-arterial injection of KCl on the development and maintenance of muscle tension during stimulation of the nerve at a high frequency. Nine dogs anesthetized with sodium barbital were used in the experiments. Both *Triceps surae* were prepared as previously described for the rat.² Spring levels were used for the recording of muscle tension. The muscles of each leg were stimulated simultaneously through the cut sciatic nerves with electrodes connected in parallel and led from a Thyatron stimulator. The stimulus strength was about 6 times threshold

and the frequency of stimulation was 275 to 300 shocks per second. The duration of stimulation was usually 5 or 10 seconds with an equal interval of rest between periods of stimulation. In some experiments stimulation was continuous during the period of injection. In a few experiments the muscles were stimulated with single shocks during the period of injection. Injections were made into the femoral artery. Two rates of injection were employed: slow, 2 to 3 mg of KCl (1.12% solution) per second; fast, 15 mg of KCl (4.2% solution) per second.

Results. During indirect stimulation at 300 shocks per second the peak of tetanic tension was followed immediately by a rapid decline of tension to a much lower level which was either maintained or slightly increased during continued stimulation (Fig. 1, 1A). Slow injection of KCl gradually reduced the rate and the extent of decline of tension in the perfused muscles during successive periods of stimulation. The maximal enhancement of tetanic tension was attained approximately one minute after the beginning of injection (Fig. 1, 1B). In the contralateral muscles, not subjected to KCl perfusion, little change in the tension curve occurred during the same interval (cf. 2A and 2B of Fig. 1). By the end of 2 minutes of slow injection

¹ Rosenblueth, A., and Cannon, W. B., *Am. J. Physiol.*, 1940, 130, 205.

² Walker, S. M., *Am. J. Physiol.*, 1947, 149, 7.

³ Walker, S. M., and Laporte, Y., *J. Neurophysiol.*, 1947, 10, 79.



Fig. 1. Myograms showing the effect of intra-arterial injection of KCl on the response of the *Triceps surae* to electric stimulation of the cut sciatic nerve. Time signal: 1-second intervals. Elevations of the time line show periods of injection.

1A to 1E: Recordings of tension from muscles perfused with 2 mg of KCl (1.12% solution) per second for 2 minutes and stimulated during alternate 5-second intervals with 300 shocks per second. 1A: Before injection. 1B: After 1 minute of injection. 1C: After 2 minutes of injection. 1D: One minute after injection was stopped. 1E: Ten minutes after injection was stopped. 2A to 2E: Recordings of tension from the contralateral muscles stimulated in parallel with the muscles recorded in 1A to 1E.

1F: Muscles perfused with 3 mg of KCl (1.12% solution) per second for 54 seconds during continuous stimulation at 275 shocks per second. 2F: The contralateral muscle stimulated in parallel.

1G: Muscles perfused with 15 mg of KCl (4.2% solution) per second for 7 seconds during continuous stimulation at 275 shocks per second. 2G: The contralateral muscles stimulated in parallel.

1H: Muscles perfused with 3 mg of KCl (1.12% solution) per second for 68 seconds during stimulation at 1 shock per 5 seconds. 2H: The contralateral muscles stimulated in parallel.

I: Muscles perfused with 15 mg of KCl (4.2% solution) per second for 40 seconds during stimulation at 1 shock per 1.5 seconds. J: The same muscles recorded in I showing partial recovery of neuromuscular transmission 20 minutes after the injection. K: The same muscles recorded in I showing complete recovery of transmission 25 minutes after the injection.

the perfused muscles showed marked depression of tetanic tension, while the response of the contralateral muscles showed only slight change (Fig. 1, 1C and 2C). At this point injection was discontinued. An increase of tetanic tension occurred in each successive period of stimulation of the muscles which had received intra-arterial injection of KCl (Fig. 1, 1D). Ten minutes after injection was stopped the tension curve was almost normal (Fig. 1, 1E).

Slow injections made during continuous stimulation at 275 shocks per second produced an increase of tension followed by a decline (Fig. 1, 1F). If injections were stopped shortly after the decline began another rise of tension occurred. If, on the other hand, injections were continued for several seconds after the decline appeared no increase of tension occurred after cessation of injection. When fast injections were given during stimulation a depression of tension be-

gan a few seconds after the start of injection. If the duration of injections was only 6 to 8 seconds an increase of tension followed shortly after the cessation of injection (Fig. 1, 1G). Longer periods of injection produced either a prolonged depression of tension or complete neuromuscular block.

The typical potentiation of muscle response to single shocks was produced by slow injections (Fig. 1, 1H). It is of interest to note that continuation of slow injection induced slight contracture which was followed shortly by diminution of response to single shocks. When injection was stopped at the onset of depression recovery of the normal response occurred within 10 to 20 seconds. Marked contracture and complete neuromuscular block were produced by prolonged fast injections (Fig. 1I). Twenty to 25 minutes were required for recovery of the response to single indirect stimuli (Fig. 1, J and K). It was shown that the quantity of KCl required to produce complete neuromuscular block did not impair the contractile strength of the muscle stimulated directly with single or with high-frequency shocks.

Discussion. Direct stimulation in the curarized muscle of the rat⁴ has shown that KCl-treatment does not increase tetanic tension. Therefore, it is assumed that all enhancement of tetanic tension observed in this study, except increases resulting from contracture, is due to improvement of neuromuscular transmission. The presence of contracture usually could be recognized by irregularities in the tension curve (Fig. 1, 1F). The slowest rate of injection of KCl (2 mg per second) produced no contracture although marked augmentation of tetanic tension occurred (cf. 1A and 1B of Fig. 1). Moreover, it seems likely that the contracture during injection of 3 mg per second of KCl pro-

duced only a small portion of the increased tension observed at a high frequency of stimulation (cf. the slight increase of tension induced by contracture in 1H with the marked increase of tension during KCl injection in 1F).

The observation that neuromuscular transmission is facilitated by intra-arterial injection of small quantities of KCl confirmed the results obtained by intraperitoneal injection of KCl in the rat.² Furthermore, the failure of the injection to induce similar changes in the contralateral muscles showed that the results were not attributable to central effects on the circulation or respiration. The observations of enhancement of neuromuscular transmission with small amounts of KCl and of depression with large amounts are in agreement with the findings in the curarized nerve-muscle preparation from the frog.³ Moreover, the present results in the dog have shown that both the augmenting effect and the depressant action of K on neuromuscular transmission occur in uncurarized muscle.

Summary. 1. Continuous slow injection of KCl (2 to 3 mg per second of 1.12% solution) produced initial enhancement and subsequent depression of neuromuscular transmission in muscle stimulated indirectly at 275 to 300 shocks per second.

2. Fast injection of KCl (15 mg per second of 4.2% solution) produced a prompt depression of neuromuscular transmission. Continued injection produced marked contracture and complete neuromuscular block.

3. Enhancement of neuromuscular transmission may occur in the absence of contracture or in the presence of mild contracture. Amounts of K sufficient to induce marked contracture usually produced complete neuromuscular block.

4. During complete neuromuscular block by KCl injection the response of the muscle to direct stimulation was unimpaired.

⁴ Walker, S. M., unpublished data.

Further Studies on the Acute Toxicity of Mercurial Diuretics.*

ROBERT A. LEHMAN.

From the Department of Therapeutics, New York University College of Medicine.

A number of reports have appeared concerning a serious and sometimes fatal reaction which has been observed following intravenous injection of therapeutic doses of mercurial diuretics.¹⁻³ This has been shown to be due to a direct action on the heart giving rise to a variety of changes in rhythm and conduction^{4,5} and a decrease in work capacity.⁶ The reaction appears to be characteristic of mercurials in general, whether organic or inorganic, and not specifically of the diuretic group.

In a previous report, the acute lethal dose was given for the common diuretics as estimated by intravenous injection in intact unanesthetized cats. This work has now been extended to determine the comparative dosages at which cardiac toxicity develops as indicated in the electrocardiogram. The drugs used in the present comparisons were:

* The investigation of the comparative cardiac toxicity of the mercurial diuretics was made with the assistance of grants from the Committee on Therapeutic Research of the Council on Pharmacy and Chemistry of the American Medical Association, from Lakeside Laboratories, Inc., Milwaukee, Wis., and from Campbell Products, Inc., New York City. The preparation and study of the pharmacology of N(γ -carboxymethylmercaptomercuri- β -methoxy)propyl camphoramic acid disodium salt was assisted by a grant from Campbell Products, Inc.

¹ DeGraff, A. C., and Nadler, J. E., *J. Am. Med. Assn.*, 1942, **119**, 1006.

² Wexler, J., and Ellis, L. B., *Am. Heart J.*, 1944, **27**, 86.

³ Pines, I., Sanabria, A., and Arriens, R. T. H., *Brit. Heart J.*, 1944, **6**, 197.

⁴ Volini, I. F., Levitt, R. O., and Martin, R. J., *J. Am. Med. Assn.*, 1945, **128**, 12.

⁵ DeGraff, A. C., and Lehman, R. A., *J. Am. Med. Assn.*, 1942, **119**, 998.

⁶ Long, W. K., and Farah, A., *J. Pharmacol. Exp. Therap.*, 1946, **88**, 388.

Mercuric Chloride: A 5.4% solution in distilled water.

Mercuzanthin: A solution of the sodium salt of N(γ -hydroxymercuri- β -methoxy)propyl camphoramic acid-theophylline (Mercurophylline Injection U.S.P. XIII).

Salyrgan-Theophylline: A solution of the sodium salt of ortho-N(γ -hydroxymercuri- β -methoxy)propyl carbamyl phenoxy acetic acid-theophylline (Mersalyl and Theophylline Injection, U.S.P. XIII).

Mercurhydrin: A solution of the sodium salt of N(γ -hydroxymercuri- β -methoxy)propyl N'(succinyl)urea and theophylline.

MIT6: A solution of the disodium salt of N(γ -carboxymethylmercaptomercuri- β -methoxy)propyl camphoramic acid. N(γ -hydroxymercuri- β -methoxy)propyl camphoramic acid was synthesized from camphor, allyl amine and mercuric acetate by methods described in the literature. A solution of the sodium salt of this acid was then added to an aqueous solution of sodium thioglycolate. The resulting solution of the mercaptide was brought to pH 7 to 8 by addition of sodium hydroxide.

Unless otherwise stated, all of the above drugs were used at a concentration of approximately 39 mg of mercury per cc.

Procedure. Normal adult cats of both sexes weighing between 2 and 4 kg were used in this study. The animals were anesthetized with 0.5 cc per kg of dial (10%) with urethane (40%) half of which was given intramuscularly and half intraperitoneally. When necessary, anesthesia was completed by cautious intravenous injection of pentobarbital sodium solution. Electrocardiograms were recorded from lead II. A control tracing was taken and the undiluted solution of the mercurial was immediately injected into the saphenous vein. The total volume was injected over 30 seconds in the case of vol-

TABLE I.
Effect of Intravenous Injection of Mercurial Diuretics on the QRS Interval.

Mercuranthin				Salyrgan-Theophylline				Mercurydrin				Mercuric Chloride			
Dose in cc per kg	Control QRS sec.	Maximum QRS observed sec.		Dose in cc per kg	Control QRS sec.	Maximum QRS observed sec.		Dose in cc per kg	Control QRS sec.	Maximum QRS observed sec.		Dose in cc per kg	Control QRS sec.	Maximum QRS observed sec.	
.035	.03	NC		.038	.04	NC		.05	.02	NC		.01*	.03	NC	
.035	.03	NC		.038	.04	NC		.05	.03	NC		.04*	.03	NC	
.071	.03	NC		.117	.03	.12		.1	.03	NC		.05	.03	.08	
.071	.02	.04		.233	.03	.05		.2	.03	.06		.1	.03	.16	
.142	.03	.20		.233	.03	.16		.2	.03	.10		.2	.02	.05	
.142	.03	.14		.456	.02	.08		.4	.02	.12					
.283	.06	.34		.456	.03	.10		.4	.02	.14					
.283	.03	.10			.03			.8	.03	.12					
.566	.02	.12							.04	.10					
.566	.03	.14													

NC...No change.
* 0.4% solution.

umes up to 0.8 cc per kg. For the large volumes required in the case of MT6, a burette was used and the solution injected at approximately 2 cc per kg per minute. Tracings were then taken at intervals of 1, 2, 3, 5, 10, 15, 20, 30, 40, 50 and 60 minutes after injection. In some cases this series was extended up to 6 hours at 15-minute intervals.

Results. The observations have been summarized in Table I. In every case the initial dose was low enough so that no change in the electrocardiogram occurred within an hour after injection. This was then increased by a factor of 2 until a lethal or severely toxic level was reached. In the case of Salyrgan-Theophylline, Mercurydrin, Mercuzanthin and mercuric chloride, the sequence of events following injection of a toxic dose was quite similar. The most significant changes appeared immediately after injection and could for the most part be attributed to depression of conduction. They usually consisted of atrioventricular dissociation or ventricular tachycardia and were invariably accompanied by a marked increase in the QRS interval. In some cases ventricular fibrillation and death ensued. Occasionally the ventricular rate progressively slowed until complete arrest. Usually, however, the electrocardiogram reverted in less than 30 minutes to a more or less regular sinus rhythm (Fig. 1 and 2). These phenomena are in general agreement with those reported by Volini⁴ for 2 patients on whom electrocardiograms were taken during the acute episode following the injection of a mercurial diuretic. Furthermore, the literature¹⁻⁴ indicates that the typical reaction in man comes on immediately after the injection. Therefore, in the absence of direct evidence, it will be assumed that the action of these drugs in the anesthetized cat is qualitatively the same as in man. In the case of MT6 no significant changes in the tracing were observed immediately in any dose used. The highest doses gave rise to a gradual depression of conduction and rate leading to cardiac arrest after a variable period. These changes are illustrated in Fig. 3. In Table II the

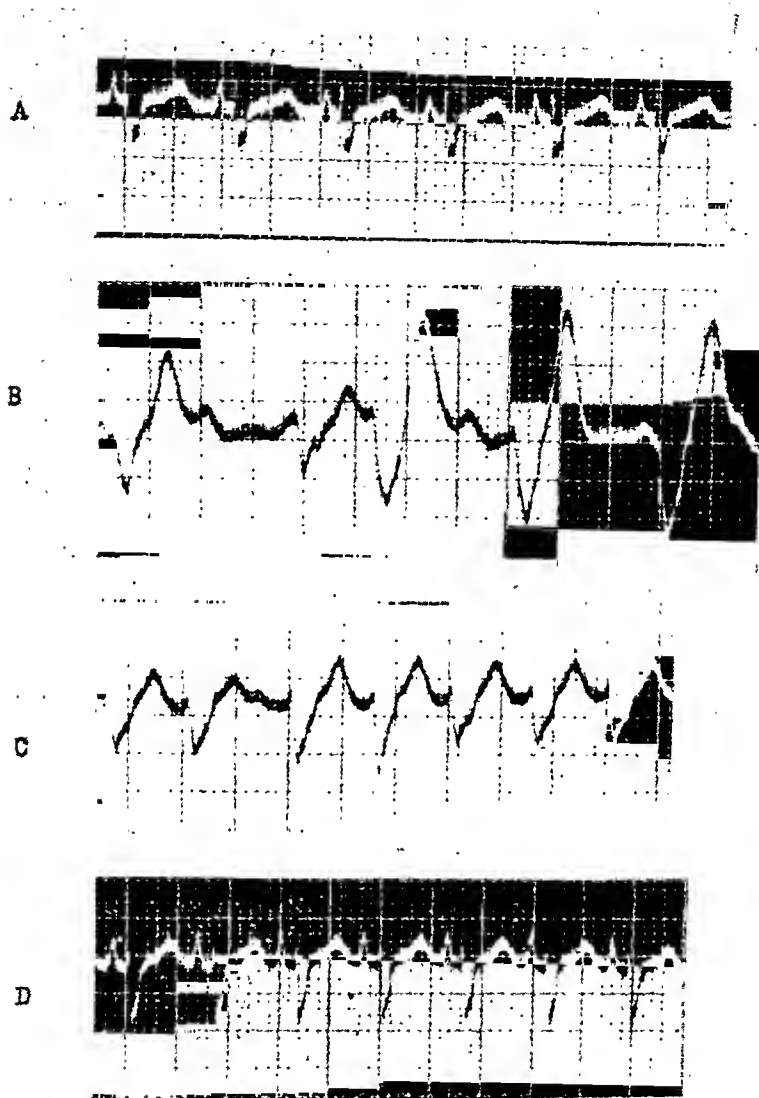


Fig. 1.

Effect of intravenous injection of 0.283 cc per kg of Mercuzanthin solution on the electrocardiogram of the anesthetized cat. A, control tracing; B, one minute after injection; C, 10 minutes after injection; D, 50 minutes after injection.

time of onset of marked electrocardiographic changes due to MT6 is given as a function of dosage. It is evident that the higher the dose the more rapidly the cardiac effects appear. However, at 4 cc per kg no cardiac toxicity appeared within 6 hours after injection. It is not to be inferred, however, that MT6 or indeed any of the mercurials used in this study can be tolerated indefinitely

ly at these large doses. The majority of the animals died, or were expected to die, after several days due to mercury intoxication.

Quantitatively, the toxic effect of the first 4 mercurials is most consistently reflected in the electrocardiogram in an increase in the QRS interval. Using this as a criterion, the maximum tolerated dose for each drug has been roughly estimated and is given in Ta-

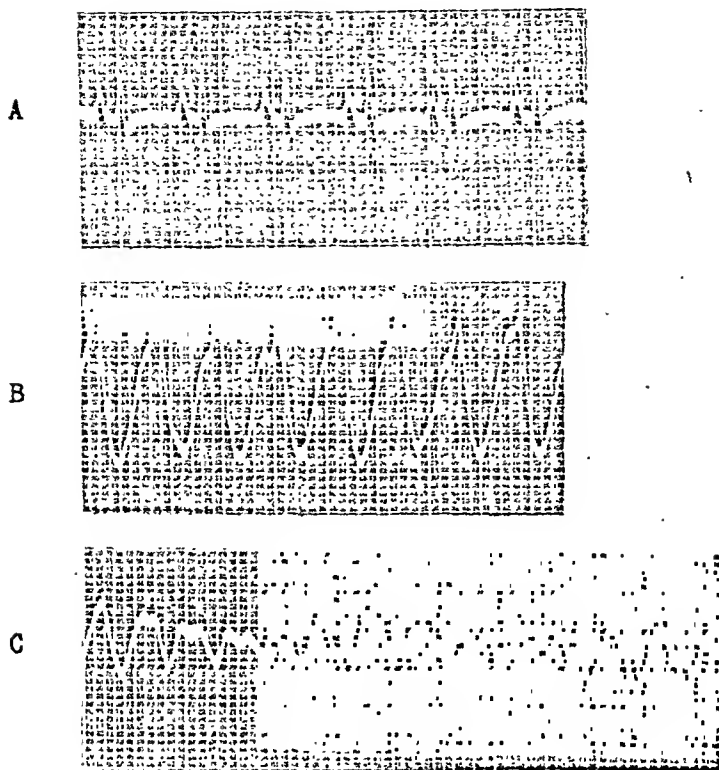


Fig. 2.

Effect of intravenous injection of 0.233 cc per kg of Salyrgan-theophylline solution on the electrocardiogram of the anesthetized cat. A, control tracing; B, one minute after injection; C, 3 minutes after injection. Death ensued shortly after C.

TABLE II.
Onset of Electrocardiographic Changes After MT6.

Dose in cc per kg	Time in minutes for onset of major E.K.G. changes (other than alterations in rate or amplitude of deflections.)	Period of observation in min
2	none	60
2	"	60
4	"	90
4	"	60
4	"	360
8	75	75
8	none	60
8	105	120
16	10	30
16	50	50
16	90	90
16	60	75

ble III. It will be noted that there is comparatively little difference in toxicity among the 3 commonly used mercurial diuretics. Furthermore, mercuric chloride, while clear-

ly the most toxic substance studied, is closer to the diuretics than might have been anticipated. The toxicity of MT6 when judged in terms of the immediate electrocardiographic changes described above is less than 1/400 that of mercuric chloride or 1/160 that of Mercuhydrin, which is otherwise the least toxic of the group.

Discussion. Johnston⁷ showed that sodium thiosulfate could restore the normal force and rhythm of the isolated turtle heart poisoned with Salyrgan or Mercuzanthin. In a previous report from this laboratory⁵ it was indicated that thiosulfate exerts a similar action in the intact cat. More recently Long and Farah⁶ have investigated the prophylactic and therapeutic action of 2 monothiol

⁷ Johnston, R. L., *J. Lab. Clin. Med.*, 1941, **27**, 303.

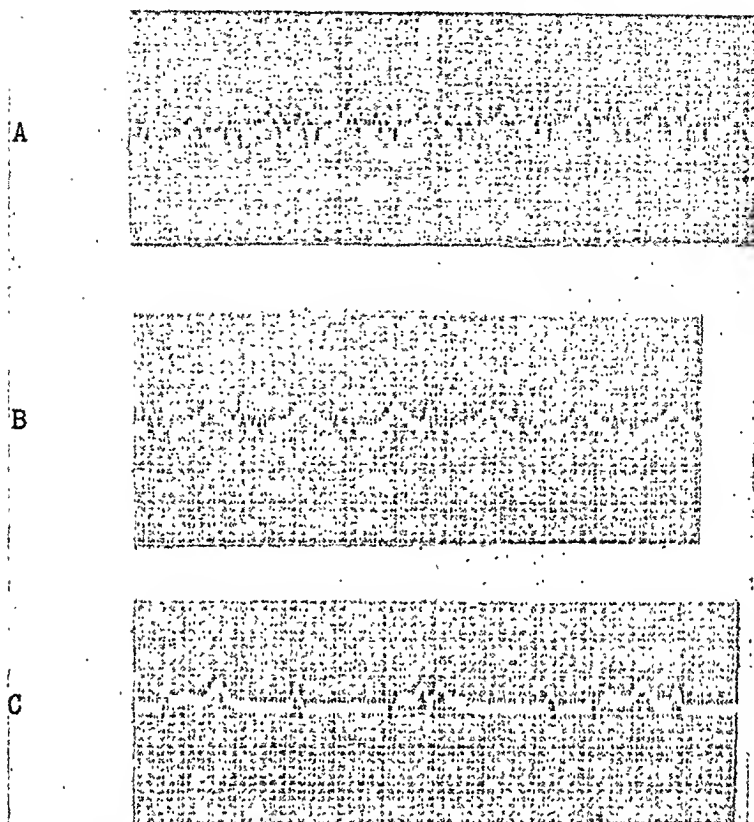


Fig. 3.

Effect of intravenous injection of 16 cc per kg of MT6 solution on the electrocardiogram of the anesthetized cat. A, control tracing; B, 40 minutes after injection; C, 50 minutes after injection. The animal died shortly after tracing C.

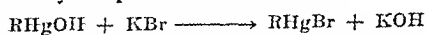
TABLE III.
Comparative Acute Cardiac Toxicity of Mercurials.

Compound	Max. tolerated intravenous dose in cc per kg based on widening of the QRS interval	Ratio
Mercuric chloride	0.04	1.0
Salyrgan-Theophylline	0.058	1.4
Mercuzanthin	0.071	1.8
Mercurydrin	0.1	2.5
MT6	>16.0	>400.0

(cysteine and glutathione) and one dithiol (2,3-dimercapto propanol, British Antilewisite) against the acute cardiac effects of Salyrgan. They found that the intravenous LD₅₀ of Salyrgan in mice was slightly increased by intravenous injection of a mol equivalent of any of the above compounds

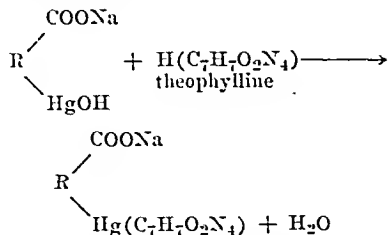
one minute before the mercurial. They also reported that arrhythmias produced in the isolated heart lung preparation of the dog as well as the intact dog could be successfully treated with these compounds. 2,3-Dimercapto propanol was considerably more effective than monothiol in the intact dog but not in the isolated heart. Apparently no attempt was made to combine the mercurial with the thiol before injection and compare its toxicity with the parent drug.

It has long been known that compounds of the general structure RHgOH will react with many simple salts as follows:

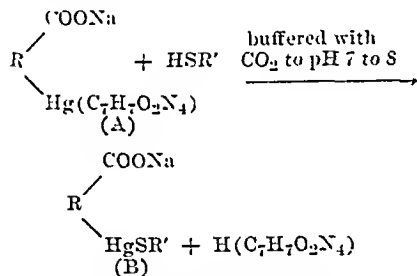


where the base formed may be quantitatively titrated. Presumably such a reaction goes

to completion because of the failure of RHgBr to ionize. The common mercurial diuretics are prepared by a similar reaction:



It has been previously shown⁸ that this combination with theophylline results in a decrease in the local toxicity of the mercurial and perhaps a slight improvement in the acute cardiac toxicity.⁵ Further experiments showed that the following reaction:



will cause the precipitation of theophylline in practically theoretical yield thus suggesting that the mercaptide is more stable than the theophylline complex. The experiments described above correspondingly demonstrate a very considerable decrease in the cardiac toxicity for a compound of structure B (MT6) as compared with compounds of structure A (Mercuhydrin, Mercuzanthin, Salyrgan-Theophylline). These results are quite out of line with those of Long and Farah⁶ in which treatment with the thiol preceded the injection of the mercurial. This might have been expected, however, because in their experiments the thiol would be required to compete for the mercurial with

whatever group the mercurial combines with in the tissues. The difference they observed in the effectiveness of 2,3-dimercapto propanol as compared with simple monothiols may then be caused by a difference in the fate of the detoxifying agent.

A number of reports have recently appeared concerning the general subject of detoxification of heavy metals by formation of mercaptides, especially in connection with the development of British Antilewisite. Most of this work is not relevant to the acute action of mercury on the heart and need not be considered here. Moreover, it should be noted that the magnitude of detoxification demonstrated in the above experiments is not necessarily capable of wide application. Thus the acute intravenous lethal doses of a series of alkyl mercuric thioglycollates (also defined by structure B) reported by Cohen⁹ are only slightly greater than the maximum tolerated doses given in Table III for the 3 diuretics.

Clinical studies with MT6 will be reported elsewhere.

Summary. Mercuric chloride, Salyrgan-theophylline, Mercuzanthin, Mercurhydrin and $\text{N}(\gamma\text{-carboxymethylmercaptomercuri-}\beta\text{-methoxy)propyl camphoramic acid disodium salt (MT6)}$ have been compared with respect to their ability to cause cardiac toxicity as manifested by changes in the electrocardiogram of the cat. The toxicity has been found to decrease in the order named. Compound MT6 causes no immediate changes in the electrocardiogram in doses up to 160 times the maximum tolerated dose of Mercurhydrin. This improvement appears to be due to the relatively greater stability of the mercaptide as compared with the theophylline complex.

The author wishes to express his thanks to Doctor A. C. DeGraff and Doctor R. C. Batterman for their advice and cooperation and to the Misses Alice Sullivan and Jean Lippold for technical assistance.

⁸ DeGraff, A. C., Batterman, R. C., and Lehman, R. A., *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 373.

⁹ Cohen, S. J., *J. Pharm. Exp. Therap.*, 1929, **35**, 343.

Effect of the Purified Blood Group A Substance on Permeability of the Capillaries.

ULRICH FRIEDEMANN, FREDERICK B. TRAUB, AND DORETT LANGSTADT.
(Introduced by Albert E. Sobel.)

From the Department of Bacteriology, The Jewish Hospital of Brooklyn.

In a previous communication,¹ it was shown with the aid of a new method that a number of substances increase the permeability of the capillaries to a very high degree. Very active were commercial peptones. Some of the constituents of commercial peptones were examined, therefore, and in the course of these investigations it was found that A and B substances of Witebsky had a strong effect on capillary permeability. It could not be decided, however, whether this effect was due to the blood substances themselves or to impurities. To answer this question we first tried to make use of the observation that enzymes in saliva and feces destroy the blood group substances.²⁻⁵ We, therefore, treated the AB substance of Witebsky with extracts of feces and the saliva of non-secretors and examined whether the effect on capillary permeability disappeared with the blood group activity. These attempts, however, were frustrated by the fact that the extracts of feces and the saliva of non-secretors themselves had a very strong effect on capillary permeability. A new avenue of approach was opened by the work of Kabat, Bendich and Bezer^{6,7} who succeeded in purifying the blood group A substance. The capillary effect of this substance will be de-

scribed in the present paper. Included are investigations on an inactive substance, likewise isolated by Kabat, which is chemically and physico-chemically indistinguishable from the A substance but differs from it serologically. The nature of this substance will be discussed below.

Methods and Materials. The method used in this investigation for the demonstration of the capillary effect is the indirect intracutaneous test with diphtheric toxin and the indirect intramuscular test with tetanic toxin. Both procedures have in common that the increased capillary permeability manifests itself in the increased effect of the antitoxin. The details of this method, and the evidence for this interpretation of the experimental results were presented in our previous communication.¹

The preparation of the purified A substance has been described by Kabat, Bendich, and Bezer.^{6,7} Gastric mucin or stomach linings of hogs were digested with pepsin and purified with the aid of the method of Morgan

TABLE I.
Capillary Effect of the Purified Blood Group A Substance as Demonstrated by the Indirect Skin Test with Diphtheric Toxin.

Dilution of toxin	Series A (control)	Series B
1:50	N	—
1:100	N	E++
1:200	E++++	E+
1:400	E+++	E+
1:800	E++	O
1:1,600	E+	O
1:3,200	E+	O
1:6,400	O	—
1:12,800	O	—

N: necrosis.

E: erythema.

Number of + indicates extent of erythema.

O: no reaction.

—: not done

Results read after 72 hr.

1 Friedemann, U., Traub, F. B., and Langstadt, D., *J. Immunol.*, 1946, **54**, 197.

2 Schiff, F., and Akune, M., *Munch Med. Wochensh.*, 1931, 657.

3 Stimpf, A., *Z. f. Immunitätsf.*, 1932, **76**, 159.

4 Schiff, F., and Weiler, G., *Biochem. z.*, 1931, **235**, 454.

5 Schiff, F., and Weiler, G., *Biochem. z.*, 1931, **239**, 489.

6 Kabat, E. A., Bendich, A., and Bezer, A. E., *J. Exp. Med.*, 1946, **83**, 477.

7 Bendich, A., Kabat, E. A., and Bezer, A. E., *J. Exp. Med.*, 1946, **83**, 485.

TABLE II.

Capillary Effect of the Purified Blood Group A Substance and of a Preparation Containing 60% A Substance and 40% "Inactive Substance" as Demonstrated by the Indirect Test with Tetanic Toxin.

Dilution of antitoxin	Series A (control)	Series B	Series C
1:20	+6, L.T. 1	—	—
1:40	+6, L.T. 1	—	L.T. 2, O
1:80	+3, +3	—	L.T. 2, O
1:160	+2, +6	L.T. 1	L.T. 2
		L.T. 2	L.T. 5
1:320	—	L.T. 1	+5, +8
		L.T. 4	
1:640	—	+2, G.T. 4	+3, +6
1:1,280	—	+5, G.T. 4	+3, +3

—: not done.

O: no symptoms.

L.T.: local tetanus.

G.T.: general tetanus.

+: indicates death

Numerals indicate day of onset of tetanus or of death.

and King.⁸ The stomachs of some hogs yielded the pure A substance while from other stomachs a second substance was obtained, chemically identical with the A substance but serologically inactive. Finally, from the pooled hog stomachs was obtained a product composed of 60% of A substance and 40% of inactive substance. These 3 preparations were kindly placed at our disposal by Dr. Kabat. In the following experiments 1% solutions of these substances in saline were used.

Experiment 1. One cc of diphtheric antitoxin 874 (1600 units per mil.) in a dilution 1:50 was injected intravenously into a white rabbit weighing 2500 g. Immediately afterward 0.1 cc of serial dilutions of diphtheric toxin 1116 were injected intracutaneously. The serial dilutions of toxin were injected in duplicates. In Series A the toxin was diluted in saline solution, in Series B it was diluted in a 1% solution of the purified A substance. The results are recorded in Table I.

It will be seen from Table I that at least 8 times more toxin has been neutralized in Series B than in Series A. This result we interpret as indicating that the purified A substance increases the permeability of the capillaries of the skin to antitoxin approximately 8 times.

Experiment 2. 0.5 cc of serial dilutions

of tetanic antitoxin 327 (1200 units per mil.) was injected intravenously into guinea pigs weighing 250 g. Twenty lethal doses of tetanic toxin 1556 (0.1 mil. of a dilution 1:25) were injected intramuscularly. The experiments were carried out 3 times. In Series A the toxin was diluted in saline solution, in Series B in a 1% solution of the purified A substance and in series C in a solution of the preparation containing 60% A substance and 40% inactive substance. The results are recorded in Table II.

Again in Series B the antitoxin is approximately 8 times more effective than in Series A. In Series B the minimal concentration which protected both animals against death was 1:320 while in Series A even a concentration 1:40 protected only one animal. In Series C the antitoxin was approximately 4 times more effective than in Series A. These results indicate that a 1% solution of the purified A substance increased the permeability of the capillaries to antitoxin about 8 times while with a 1% solution of the preparation containing 60% A substance and 40% "inactive" substance the increase was about 4 times.

Experiment 3. One cc of diphtheric antitoxin 874 (1600 units per mil.) was injected intravenously into a white rabbit weighing 2500 g. 0.1 cc of serial dilutions of toxin 1116 was injected intracutaneously. In Series A the toxin was diluted in saline, in

⁸ Morgan, W. T. J., and King, H. R., *Biochem. J.*, 1943, 37, 640.

TABLE III.
Capillary Effect of the "Inactive Substance" as
Demonstrated by the Indirect Skin Test with
Diphtheric Toxin.

Dilutions of toxin	Series A (control)	Series B
1:50	N	—
1:100	N	N
1:200	E++++	E++++
1:400	E+++	E+++
1:800	E++	E+
1:1,600	E+	O
1:3,200	E+	O
1:6,400	O	—
1:12,800	O	—

N: necrosis.

E: erythema.

No. of + indicates extent of erythema.

O: no reaction.

—: not done.

Result read after 72 hr.

Series B in a 1% solution of the "inactive" substance. The results are recorded in Table III.

As may be seen from Table III, the inactive substance increases the permeability of the capillaries 4 times.

Discussion. The scarcity of the material at our disposal made experiments on a larger scale impossible. The quantitative data, therefore, require confirmation when larger quantities of the purified blood group substances will be available. The indirect intracutaneous test with diphtheric toxin as well as the indirect test with tetanic toxin, however, leaves no doubt that the purified A substance increases the rate of neutralization of toxins, presumably resulting from the increase of the permeability of the capillaries. The fact that the "inactive" substance likewise increased the rate of neutralization and thus presumably had affected the capillary permeability, although to a lesser degree, seems at first to detract from the significance of our findings. Dr. Kabat, however, has authorized us to publish the following personal communication:

9 Personal communication.

"Kabat, Bendich and Bezer⁹ have found that Type XIV antipneumococcal horse serum, which has been shown to agglutinate cells of groups A, B and O,¹⁰ gives precipitin reactions with both the active and the inactive hog substances, leading them to the inference that the inactive substance may be associated with blood group O activity." It is highly probable, therefore, that the capillary effect of the "inactive" substance is due to its relationship to the blood group O substance. The blood group B substance is not available in a purified form and nothing can be said, thus far, as to its effect.

As shown in our previous communication,¹ the capacity of increasing capillary permeability is common to a wide variety of substances. To these we now have added the purified blood group substances. This finding assumes additional significance in view of the presence of these substances in the tissue fluids of secretors. Schiff,¹¹ and Friedenreich and Hartman¹² have discussed the question whether these substances may fulfill any physiological function. On the basis of the results reported in the present paper, the possibility must be considered that they may play a role in the regulation of capillary permeability. From this point of view the division of human beings into secretors and nonsecretors gains added interest.

Summary. With the aid of a new method it was found that the purified blood group A substance considerably increases capillary permeability. Similar results were obtained with the "inactive" substance purified by Kabat *et al.* from hog stomach, and probably closely related to the group O substance.

10 Weil, A. J., and Sherman, E., *J. Immunol.*, 1939, 30, 139.

11 Schiff, F., *Über die blutgruppenspezifischen Substanzen des menschlichen Körpers*, Gustav Fischer, Jena, 1931.

12 Friedenreich, V., and Hartmann, G., *Z. f. Immunitätsf.*, 1938, 92, 141.

Reaction of the Human Body Louse: (*Pediculus humanus corporis*) to the Ingestion of Guinea Pig Blood.

VICTOR CABASSO.* (Introduced by Herald R. Cox.)

From the Section of Viral and Rickettsial Research, Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

There seems to be no doubt that man apparently is the only natural host of the body louse. Human body lice have occasionally been found on domestic animals but, as Nuttall noted¹ ". . . this fact has the same importance as finding a louse on the hearth-rug."

Charles Nicolle² on the other hand, in experimental typhus fever studies, had no difficulty in feeding lice repeatedly on monkeys (*Macacus rhesus*) and found that a certain proportion of lice when fed on monkeys will remain alive for as long as 20 days. However, Bacot and Segal³ found only rare survivors among lice fed on monkeys for 4 successive days.

While some successful attempts have been reported of feeding body lice on rabbits, guinea pigs, mice, fowl, pigeons, etc., many more failures are recorded. It has often been claimed that blood of certain animals (particularly of rodents) is toxic to the genus *Pediculus* but, as Buxton noted,⁴ no one yet has conducted experiments comparing the longevity of lice given a single blood meal on guinea pigs or rats, or on man, to that of unfed lice.

Thus, the possibility exists that the blood of animals, other than of man, is not toxic to the body louse, but that the body louse is able to digest human blood because its digestive enzymes are specific only for human red blood cells.

The following experiments, conducted at

* Associate member of Pasteur Institute of Tunis.

¹ Nuttall, G. H. F., *Parasitology*, 1917, **10**, 80.

² Nicolle, C., *Ann. Inst. Pasteur*, 1910, **24**, 243.

³ Bacot, A. W., and Segal, J., *Brit. J. Path.*, 1922, **3**, 125.

⁴ Buxton, P. A., *The Louse*, 1939, 2nd edition, Edward Arnolds, London.

the Pasteur Institute of Tunis, seem to confirm this hypothesis. Only one species of rodent, the guinea pig was used.

Experiment I: One hundred female lice from the stock laboratory colony were divided into 2 lots of 50 lice each (A and B). The lice were placed in an incubator at 30-32°C, and kept unfed for 30 hours in order to clear their intestines of the last human blood-meal. Lice of Lot A then were fed on the arm of a human volunteer while lice of Lot B were fed on the shaven and cleansed flank of a guinea pig. After feeding for one hour, the lice of both lots were seen to be fully engorged with blood, as indicated by the large red spot in their mid-gut visible through the integument. The lice of both lots were then returned to the incubator (30-32°C). At hourly intervals thereafter a few of the lice were withdrawn from the incubator, dissected in a drop of saline and examined microscopically with the following results:

Lot A. After one hour the lice showed a great number of intact red blood cells although it was observed that some red cells already had been destroyed as evidenced by the presence of a chocolate-brown amorphous mass. After 2 hours the number of intact red blood cells was decreased still further and by the end of 4 hours all the red cells had been destroyed. The spot in the mid-gut at this period appeared dark chocolate-brown, or almost black in color.

Lot B. The red blood cells remained intact in appearance until the death of the lice which generally took place about 48 hours after the ingestion of guinea pig blood. The intestines were still full of unchanged blood. A large number of lice died showing signs of ruptured intestines or radically altered permeability of the intestines. The red color,

limited at first to the intestines, diffused progressively into the abdominal cavity, into the legs and even into the extremities of the antennae until the entire louse was red in color with a darker spot present in the region of the mid-gut. This phenomenon occurs to a certain extent in normal louse colonies⁴ but appears rather more frequently among lice fed on guinea pig blood.

The above observations indicate that it is apparently impossible for the body louse to digest guinea pig red blood cells. It is believed that the intestines are ruptured by their exertions to destroy or eliminate the guinea pig red blood cells.

Experiment II. Three lots of 30 lice each (15 males and 15 females) 12-15 days of age, were isolated in separate boxes marked A, B and C. The boxes were placed in an incubator at 30-32°C, and the lice were held unfed for an average period of 36 hours. At the end of that time the lice were devoid of blood as could be seen through the integument. The following experiments were then performed:

Box A. The lice were fed on man for one hour. They were then returned to the incubator and held without further feeding until death occurred, as follows:

- 24 hrs.—29 lice alive; all fully engorged
(1 male died prior to feeding)
- 48 " —29 lice alive; digestion of the blood meal completed
- 72 " —2 males and 2 females dead; 25 lice alive, intestines completely empty
- 96 " —no changes observed
- 120 " —all lice dead

Box B. These lice were fed on the shaven, cleansed flank of a guinea pig for one hour, and then stored unfed in the incubator (30-32°C) until death occurred as follows:

- 24 hrs.—30 lice alive; all fully engorged; no visible digestion of blood apparent
- 48 " —2 males and 4 females dead, the remaining lice move with difficulty; a large number show signs of ruptured intestines
- 72 " —all lice dead; the intestines are

still filled with blood of normal appearance

Box C. These lice which had already been starved for an average period of 36 hours, were held unfed in the incubator (30-32°C) until death occurred, as follows:

- 24 hrs.—30 lice alive; intestines completely empty
- 48 " —1 female and 4 males dead
- 72 " —all lice dead

An analysis of the above data indicates that: (1) Lice starved for an average of 36 hours, then fed only once on man, remain alive for approximately 4 additional days. This is the normal survival time of unfed lice under optimal conditions. (2) Lice starved for 36 hours following a human blood meal, and then allowed to feed once on a guinea pig, all die by the third day after ingestion of the guinea pig blood. Hence the time elapsed from the last human blood meal to the death of the experimental lice is approximately 4 to 4½ days. Thus, the total survival time of the lice (dating from the time of the last human blood meal) which fed on guinea pig blood (Box B) is approximately the same as that of lice which were starved for the same length of time but then fed a single time on human blood (Box A), or not fed at all (Box C).

Experiment III. Fifty 13-day-old lice were isolated in a box and held unfed in an incubator (30-32°C) for 33 hours. One died during this period. The remaining 49 lice were fed on a guinea pig for one hour and when fully engorged were placed in an incubator at 37°C for 2 hours. The purpose of this was to induce rapid dehydration and reduction of the guinea pig blood meal, permitting immediate feeding of the lice upon their withdrawal from the incubator. They were then fed on man for one-half hour. Subsequent half-hour human blood feedings were continued twice daily, morning and evening. All lice in this experiment remained alive until natural death occurred on the 35th to 40th day.

The above observations seem to confirm the hypothesis that guinea pig blood *per se* is not toxic to the body louse, since lice that have fed once on a guinea pig live their nor-

mal life span when they are allowed to continue their daily feedings on human blood.

Summary and Conclusions. Contrary to general belief, the experiments reported above indicate that guinea pig blood *per se* is not toxic to the human body louse.

In the first experiment it was demonstrated that the red blood cells of guinea pigs remain undigested in the gut of the louse.

In the second experiment it was shown that lice starved for an average period of 36 hours and then fed once on a guinea pig,

die within 72 hours, apparently of starvation. Control lice fed only on human blood, likewise were found to die 4 to 4½ days following their last human blood meal.

In the third experiment it was shown that lice fed once on guinea pig blood live their normal life span when their daily feedings are continued subsequently on human blood.

It would appear that human body lice secrete a digestive enzyme specific only for human red blood cells which makes it impossible for them to digest guinea pig blood.

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Mass Infection of Body Lice with *Rickettsia prowazeki*.

VICTOR CABASSO.* (Introduced by Herald R. Cox.)

From the Section of Viral and Rickettsial Research, Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

In a preceding article¹ it was reported that body lice (*Pediculus humanus corporis*) which have engorged on guinea pig blood are unaffected and remain alive for their normal life span if subsequent feedings are continued almost immediately (within 2 hours) on human blood. As a result of this observation, infection of human body lice with *Rickettsia prowazeki* was achieved on a large scale, following a single meal on an infected guinea pig.

The inconveniences and disadvantages of infecting lice by the rectal route, the method employed by Weigl² for the preparation of typhus vaccine, are well known. Infection of body lice by feeding them on human typhus cases, while feasible, is nonetheless rather impractical. Guinea pigs, on the other hand, may be easily infected and maintained in the laboratory. The advantages of feeding lice on infected guinea pigs, followed by feeding the lice subsequently on *typhus immune*

humans, for the ultimate preparation of typhus vaccine are obvious.

Method of infection. Fifty female lice 12-13 days of age were starved for 30 hours. These lice, as could be seen through the integument, were devoid of blood. They were then allowed to feed on a guinea pig infected 10 days previously with the Tunis strain of classical louse-borne typhus.

The flank of the guinea pig had been shaved and rubbed with alcohol, as lice often refuse to feed if the guinea pig is not prepared in this manner. In 30 minutes the lice were fully engorged, as indicated by the large red spot visible in the mid-gut.

The lice were then placed for 2 hours in an incubator at 37°C, in order to induce rapid dehydration and reduction in volume of the engorged guinea pig blood. They were then allowed to feed on a typhus-immune man for one-half hour. Subsequent human blood feedings on a typhus-immune individual were continued twice daily. By the end of the 8th day, 37 of the 50 lice were still alive, while 13 had succumbed.

A number of the surviving lice were dissected and preparations of their intestines were stained with a mixture of May-Grün-

* Associate member of Pasteur Institute of Tunis.

¹ Cabasso, V., PROC. SOC. EXP. BIOL. AND MED. 1947, 64, 437.

² Weigl, R., Arch. Inst. Pasteur Tunis, 1933, 22, 315.

wald-Giemsa. Microscopic examination of these preparations showed, without exception, rickettsiae in large numbers. The following experiments were carried out to identify the rickettsiae present.

Experiment I. Excreta of the infected lice, collected between the 8th and 10th days following the infective meal, were suspended in saline and inoculated intraperitoneally into 4 guinea pigs (51B, 58N, 62N and 64N). Three guinea pigs showed signs of classical typhus 8 to 13 days following the inoculation; the fourth (64N) died prematurely.

Forty days after the infective inoculation, guinea pigs 51B and 58N were immune to a challenge inoculation with a guinea pig brain suspension of the Tunis strain of typhus virus. Guinea pig 62N was sacrificed on the 10th day; the brain was triturated, suspended in saline, and injected intraperitoneally into guinea pigs 72N and 73N. Both of these latter guinea pigs showed typical clinical signs of typhus beginning on the 6th day and were immune to the challenge inoculation given 40 days later.

Experiment II. The intestines of 10 infected lice were triturated, suspended in saline and inoculated intraperitoneally into guinea pigs 68N and 69N. Both animals showed signs of classical typhus on the 6th day.

Guinea pig 68N was sacrificed on the 10th day and a suspension of its brain inoculated intraperitoneally into guinea pigs 74N and 75N. Both showed signs of classical typhus on the 6th and 7th days respectively, and were immune to the challenge inoculation on the 40th day.

Experiment III. Ten intact lice were sterilized externally with 70% alcohol, ground in a mortar and suspended in saline. The suspension was inoculated intraperitoneally

into guinea pigs 50B, 52B, 65N and 66N. On the 2nd day following inoculation all 4 guinea pigs showed severe signs of typhus and all showed signs of pronounced cachexia at the end of the 5th day.

Guinea pig 50B was sacrificed on the 10th day and a suspension of its brain inoculated intraperitoneally into guinea pigs 55N and 56N. Guinea pig 55N died prematurely while 56N showed signs of typhus on the 7th day and was sacrificed on the 12th day. A suspension of its brain was inoculated intraperitoneally into guinea pigs 60N and 61N, both of which showed signs of typhus on the 6th day and were immune to the challenge inoculation on the 40th day.

Guinea pigs 65N, 66N and 52B died on the 7th, 7th and 16th days, respectively, following inoculation. On autopsy the organs showed no abnormal appearance except that the viscera were covered with a glossy white, membranous-like film. Smears of this material when stained with Giemsa showed innumerable rickettsiae, both free or enclosed in protoplasm of endothelial cells.

The virus which proved to be extremely virulent on direct inoculation of infected lice into guinea pigs, assumed its normal characteristics with the usual incubation period on the first passage from guinea pig to guinea pig.

Summary and Conclusions. The experiments reported above indicate that human body lice fed once on a typhus-infected guinea pig at the height of the febrile period, and subsequently fed on a typhus-immune human, develop massive infection with *Rickettsia prowazeki*. In comparison to the rectal inoculation of lice for the preparation of the Weigl type typhus vaccine, this method of infecting lice seems to offer certain advantages.

Response of Anemic Chicks to Pteroylglutamic Acid.*

E. I. ROBERTSON, GRACE F. FIALA, M. L. SCOTT, L. C. NORRIS, AND G. F. HEUSER.

From the Agricultural Experiment Station and the School of Nutrition, Cornell University, Ithaca, N. Y.

In the course of studying the quantitative requirements of chicks for pteroylglutamic acid (folic acid),¹ information was desired regarding the ability of anemic chicks to make effective use of this substance in order to compare their response to that of anemic monkeys reported by Day and associates,² and that of human subjects described by Spies and co-workers.³

Experimental and Results. *Exp. 1.* Surviving chicks of several experiments were divided at random into 2 groups of 12 chicks each. These chicks had been reared to 4 weeks of age on Diet 653 described by Hill and associates.⁴ Microbiological assays indicated that the diet contained about 2 μ g of free pteroylglutamic acid and about 3 μ g in the conjugate form per 100 g of diet. The hemoglobin levels of these chicks averaged 2.7 g per 100 cc of blood with a range of 0.6 to 6.2 g per 100 cc.

The chicks in one group were injected intramuscularly with 100 μ g of pteroylglutamic acid;[†] those in the other group were maintained as controls. Hemoglobin values and percentage of reticulocytes were determined

at 2, 4, 6, 9, 11, 13 and 16 days following the single injection of pteroylglutamic acid.

In the group receiving the vitamin, 3 chicks died the day injections were made but no other mortality occurred during the experimental period. In the control group, 6 chicks died during the first 2 days and only 2 were alive at the end of the experiment.

The percentage of reticulocytes was strikingly greater in the group receiving the pteroylglutamic acid. Reticulocytosis reached a peak on the 6th day following injection after which it subsided rapidly to less than 10%, which is the level usually observed in untreated chicks. The peak of reticulocytosis in all cases was reached before the 9th day following the injection. These relationships are shown in Fig. 1.

Reticulocytes increased slightly in the surviving chicks in the control group. This increase may have been due to mobilization of pteroylglutamic acid in the catabolism of body tissues, or it may have been the result of normal erythropoietic stimuli occurring in this severe stage of anemia, analogous to spontaneous remission in pernicious anemia in humans.

The hemoglobin response was no less striking than the reticulocyte increase. Two days following the injection of pteroylglutamic acid, hemoglobin values had increased in most chicks. The increase was definite in all chicks by the 6th day. The peak in hemoglobin level was reached in 9 days, coincidental with the rapid decrease in reticulocytes.

Exp. 2. The purpose of this experiment was to compare oral administration with intramuscular injection of pteroylglutamic acid, and to establish the amount of the vitamin required for optimum reticulocytosis and hemoglobin formation. Seventy chicks which had been reared to 4 weeks of age on

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¹ Robertson, E. I., Daniel, Louise J., Farmer, Florence A., Norris, L. C., and Heuser, G. F., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 97.

² Day, P. L., Mims, V., and Totter, J. H., *J. Biol. Chem.*, 1945, **161**, 45.

³ Spies, T. D., Vilter, C. F., Koch, M. B., and Caldwell, M. H., *So. Med. J.*, 1945, **38**, 707.

⁴ Hill, F. W., Norris, L. C., and Heuser, G. F., *J. Nutrition*, 1944, **28**, 175.

[†] The authors are indebted to Lederle Laboratories, Inc., Pearl River, N. Y., for pteroylglutamic acid.

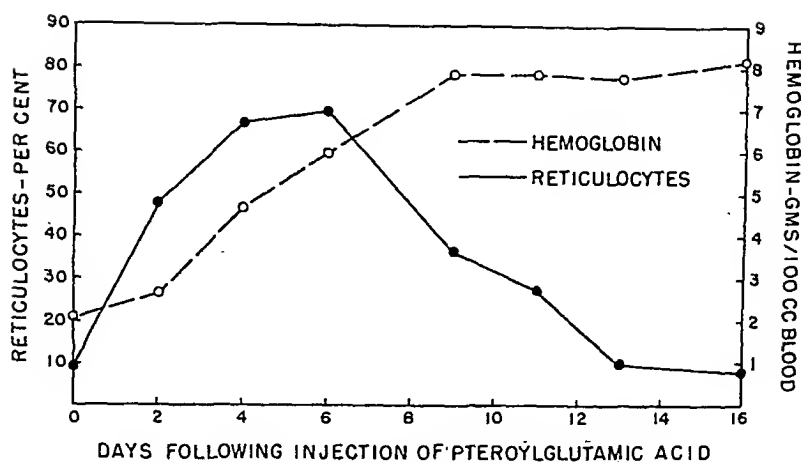


Fig. 1.

Reticulocyte and hemoglobin response of chicks to a single injection of pteroylglutamic acid.

TABLE I.
Response of Chicks to Pteroylglutamic Acid.

Groups	Days following treatment									
	0	4	7	11	0	4	7	11	4	7
	Body wt (g)				Hb level (g/100 ml)				Retics. (%)	
Basal	114	112	108	—(0)†	1.7	0.7	1.2	—	11	20
In feed*	109	129	168	220(4)	3.1	3.2	6.4	7.3	60	47
Inj. with:										
5 µg	115	143	140	163(3)	2.1	2.2	3.2	3.7	40	25
10	113	144	128	155(4)	2.4	2.8	4.1	3.4	42	26
20	109	131	148	159(5)	2.6	2.7	4.1	3.0	59	35
30	117	156	179	198(5)	2.2	3.2	5.0	6.1	63	41
40	117	136	131	153(3)	2.1	3.4	6.2	7.3	72	17
50	130	165	174	192(5)	2.3	4.4	7.5	8.0	73	39
Oral:										
5	109	131	129	132(4)	2.2	1.6	1.4	1.3	42	11
10	125	130	145	157(3)	2.8	1.4	1.7	1.8	20	25
20	118	131	131	145(3)	2.2	1.9	2.7	2.8	41	39
30	122	147	153	162(3)	1.9	2.8	3.4	3.0	56	39
40	117	144	154	156(5)	2.7	3.7	3.8	3.5	51	26
50	114	138	146	161(4)	1.9	3.5	4.6	4.0	75	39

* 500 µg of folic acid per 100 g of feed from 0 to 7 days; 250 µg thereafter.

† Numbers in parentheses indicate surviving chicks.

Diet 653 but without pteroylglutamic acid, and whose hemoglobin levels ranged between 1 and 4 g per 100 cc of blood, with weights between 100 and 150 g, were divided into 14 groups of 5 chicks each. Six groups were used for oral administration, and 6 groups for injection of pteroylglutamic acid. Of the remaining groups one was maintained as control and the other received the vitamin in its diet.

The results of Exp. 2 are presented in

Table I. Reticulocytosis was greater on the 4th than on the 7th day following treatment. This applies to the chicks which received pteroylglutamic acid in their diet, and to those to which the vitamin was administered orally or by intramuscular injection.

Hemoglobin levels were greatest in the groups which received pteroylglutamic acid by injection. When the vitamin was supplied in the feed it produced a greater response in the hemoglobin level than when admin-

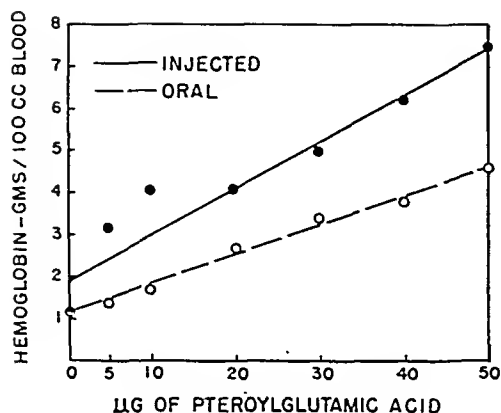


Fig. 2.

The hemoglobin response of chicks to varying levels of pteroylglutamic acid.

istered orally but separate from the feed. In all cases the response was proportional to the amount of pteroylglutamic acid administered. In the oral and injected groups the hemoglobin response was more pronounced at 7 than at 4 days following treatment. The values for the 11th day were variable due to the loss of some chicks. Hemoglobin increases after 7 days were plotted and the results are shown in Fig. 2.

Intramuscular injection of pteroylglutamic acid was approximately twice as effective in increasing hemoglobin as the same amount of the vitamin given orally. This may be due to actual destruction during the digestive processes, or to limited absorption of the vitamin.

The chicks receiving a diet containing 250 to 500 μ g pteroylglutamic acid per 100 g of diet did not show increases in hemoglobin as great as that produced by the injection of 50 μ g of the vitamin. Based upon feed consumption during the 7-day period the average intake per chick was estimated to be 486 μ g of pteroylglutamic acid. The response of these chicks in hemoglobin increase was similar to that produced by the injection of 40 μ g of the vitamin.

Discussion. The data reported for anemic

chicks indicate that their response to pteroylglutamic acid is similar to that of other species. Spies and associates³ reported peaks in reticulocyte response in 6 to 8 days following intravenous injection of folic acid in humans afflicted with macrocytic anemia in relapse. The peak of reticulocytosis rarely reached 20%. Day and co-workers² observed a 7 to 47% increase in reticulocytes between 4 and 7 days following the administration of *Lactobacillus casei* factor to vitamin M-deficient monkeys. The data reported here for anemic chicks show that reticulocyte response reached a peak 4 to 6 days following administration, and often exceeded 90% for individuals. It appears, therefore, that pteroylglutamic acid stimulates reticulocytosis in the chick the same as has been reported for man and monkey.

These data indicate that intramuscular injection of pteroylglutamic acid was more effective than oral administration in increasing the hemoglobin levels of anemic chicks. However, Goldsmith⁵ found oral administration equally or more effective in increasing hemoglobin levels of humans with macrocytic anemia. A possible explanation of this difference may be that suboptimum amounts of the vitamin were given to the anemic chicks.

Summary. Chicks deprived of pteroylglutamic acid (folic acid) for the first 4 weeks of life respond dramatically to a single administration of the vitamin given either orally or intramuscularly. The reticulocyte response preceded hemoglobin increase and reached a peak 4 to 6 days following administration. Intramuscular injection of pteroylglutamic acid was more than twice as effective as the same amount administered orally. The hemoglobin increase in 7 days was proportional to the amount of pteroylglutamic acid administered either by mouth or by intramuscular injection.

⁵ Goldsmith, Grace A., PROC. SOC. EXP. BIOL. AND MED., 1947, 64, 115.

Stability of Thrombin in the Presence of Fibrinolysin.*

ANTHONY J. GLAZKO.

From the Department of Biochemistry, Emory University, Georgia.[†]

Under certain conditions blood shows marked proteolytic activity, and digests such substrates as casein, gelatine and fibrin.¹ It has recently been shown that the proteolytic activity of serum is due principally to an endopeptidase which is homospecific with an enzymatic component of papain.² Earlier work has demonstrated the existence of an enzyme precursor ("profibrinolysin") in blood, which is spontaneously activated following the destruction of an inhibitor by treatment with chloroform or certain other denaturing agents.^{3,4} It is also activated specifically by a substance found in streptococcal cultures which is known as streptokinase.⁵ To avoid confusion of terminology, the nomenclature suggested by Loomis, George and Ryder⁶ has been used throughout this paper. The effect of trypsin on the clotting activity of thrombin was studied by Glazko and Ferguson^{7,8} prior to the recognition of fibrinolysin as an important blood protease. The work presented here demonstrates clearly that fibrinolysin has no appreciable effect on thrombin, confirming statements made by others.^{6,9} In addition

it was observed that the thrombin preparation used in these experiments had some stabilizing effect on fibrinolysin. These results are of interest because of the close association of thrombin with fibrinolysin in globulin fraction III-3,¹⁰ and of prothrombin with profibrinolysin in globulin fraction III-2.¹¹

Materials and Methods. In studying thrombin stability, serial dilutions of thrombin were made in 5 ml volumes of buffered saline, containing M/100 phosphate at pH 7.4 and a small amount of phenyl mercuric borate to prevent bacterial growth. The thrombin preparation was obtained commercially as Lederle's "Hemostatic Clotting Globulin." After warming to 38°C in a water bath, 1 ml portions of enzyme solution were added to each thrombin dilution, and the mixtures were maintained at 38°C. Clotting tests were performed at regular intervals by removing 0.5 ml of each mixture and transferring to 1.0 ml portions of 0.15% fibrinogen solution at 25°C. Clotting times were measured from the moment of addition of thrombin to the first appearance of a clot.

The stability of fibrinolysin in the presence of thrombin was estimated by measurement of the time required to lyse a standard fibrin clot. Equal volumes of thrombin and fibrinolysin solutions were warmed to 38°C, mixed and tested at intervals for fibrinolytic activity by transferring 0.5 ml amounts to 0.2 ml of fresh 1:10 thrombin, and then adding 1.0 ml of saline and 0.5 ml of 0.6% fibrinogen solution. Lytic times were measured from the time of addition of fibrinogen to the time at which the gel structure of the

* Aided by a grant from the Life Insurance Medical Research Fund.

[†] Present address: Parke, Davis and Co., Detroit.

¹ Christensen, L. R., *J. Gen. Physiol.*, 1945, **28**, 363.

² Fruton, J. S., *J. Biol. Chem.*, 1946, **166**, 721.

³ Tagnon, H. J., *J. Lab. Clin. Med.*, 1942, **27**, 1119.

⁴ Christensen, L. R., *J. Gen. Physiol.*, 1946, **30**, 149.

⁵ Christensen, L. R., and MacLeod, C. M., *J. Gen. Physiol.*, 1945, **28**, 559.

⁶ Loomis, E. C., George C., and Ryder, A., *Arch. Biochem.*, 1947, **12**, 1.

⁷ Glazko, A. J., and Ferguson, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1940, **45**, 43.

⁸ Glazko, A. J., and Ferguson, J. H., *J. Gen. Physiol.*, 1940, **24**, 169.

⁹ Seggers, W. H., and Loomis, E. C., *Science*, 1946, **104**, 461.

¹⁰ Glazko, A. J., *J. Clin. Invest.*, 1947, (May), in press.

¹¹ Edsall, J. T., Ferry, R. M., and Armstrong, S. H., *J. Clin. Invest.*, 1944, **23**, 557.

TABLE I.
Stability of Thrombin in the Presence of Globulin Fraction III-3.

Thrombin dilution	Incubation time (hr)							
	No protease added				With protease added			
	0	1	5	20	0	1	5	20
1:2	4	4	4	4	5	5	5	5
1:4	5	5	5	5	6	7	7	7
1:8	7	7	7	7	9	8	9	8
1:16	9	10	11	13	12	13	13	14
1:32	13	14	16	19	17	16	17	20
1:64	21	23	25	33	20	19	23	28
1:128	34	37	45	67	24	25	28	43
1:256	75	70	85	170	25	25	30	65
No thrombin added	—	—	—	—	30	32	48	40

Body of table gives clotting times in seconds.

Incubation mixture: 5 ml thrombin + 1 ml (= 10 mg) globulin III-3.

Clotting test: 0.5 ml incubation mixture + 1.0 ml 0.15% fibrinogen.

TABLE II.
Stability of Thrombin in the Presence of Fibrinolysin.

Thrombin dilution	Incubation time (hr)									
	No enzyme added					Fibrinolysin added				
	0	1	3.5	21	46	0	1	3.5	21	46
1:2	5	5	5	5	5	5	5	5	5	5
1:4	6	6	6	6	6	6	6	6	6	6
1:8	8	8	8	9	9	8	8	8	9	8
1:16	13	13	13	13	14	12	13	13	13	13
1:32	20	20	21	22	22	20	22	22	24	25
1:64	30	35	39	42	46	30	34	38	42	44
1:128	55	57	70	77	77	50	52	55	65	72
1:256	85	90	120	140	180	85	100	100	125	150
No thrombin added	—	—	—	—	—	—	—	—	—	—

Body of table gives clotting times in seconds.

Incubation mixture: 5 ml thrombin + 1 ml (= 1 mg) fibrinolysin.

Clotting test: 0.5 ml incubation mixture + 1.0 ml 0.15% fibrinogen.

clot broke down on gently tilting the tube.

The fibrinogen, prepared from beef blood, was obtained through the courtesy of Dr. J. D. Porsche of Armour and Company. Negative tests were obtained for the presence of prothrombin, and no lytic activity appeared when the fibrinogen was treated with an active streptokinase preparation.

Two different sources of fibrinolysin were used in these experiments. Globulin fraction III-3⁷ was first used as a convenient source of the protease, although it was later found to contain appreciable amounts of free thrombin.¹⁰ A second source of enzyme was

the more highly purified fibrinolysin described by Loomis *et al.*,⁶ which gave negative tests for thrombic activity. This was used in a buffered saline solution containing 1 mg of the dry preparation per ml.

Results. Table I presents the results of an experiment in which the stability of thrombin was studied in the presence of globulin fraction III-3. The clotting times were found to remain fairly constant with the more concentrated thrombin solutions; but dilute solutions showed some increase in clotting time. However, there were no significant differences in the rate of increase in the clotting times between the controls and the samples containing added protease.⁸ The samples to which globulin fraction III-3 had been added showed some additional

⁷ Obtained through the courtesy of Dr. John T. Edsall, and prepared under a contract between the OSRD and Harvard University from human blood collected by the American Red Cross.

TABLE III.

Fibrinolytic Activity of Globulin Fraction III-3
After Incubation with Thrombin at 38°C.

Thrombin dilution	Incubation time (hr)		
	0	1	2
1:1	7'	17'	53'
1:2	6'	13'	46'
1:4	6'	12'	46'
1:8	5'	23'	>4°
No thrombin added	5'	4°38'	>4°

Body of table gives lytic times in hours (°) and minutes (').

Incubation mixture: 2 ml thrombin + 2 ml (= 2 mg) globulin III-3.

Fibrinolysis test: 0.5 ml incubation mixture + 1 ml saline + 0.2 ml 1:10 thrombin + 1 ml 0.6% fibrinogen.

thrombic activity due to the presence of a small amount of thrombin in this fraction.¹⁰

Table II shows the results of a similar experiment carried out with the fibrinolysin preparation. Here again it is evident that the stability of thrombin was not affected by the presence of fibrinolysin. All of the clots obtained at the 21-hour testing period were completely lysed within 24 hours; and those

obtained at the 46-hour testing period were completely lysed within 54 hours, showing that active fibrinolysin was still present. However, the mixtures containing the highest concentrations of thrombin retained the greatest amount of fibrinolytic activity. The shortest lytic time (after the 46-hour incubation period) was 4 hours for the 1:2 dilution of thrombin; 9 hours for the next higher dilution; and progressively longer lytic times were observed up to a maximum of 54 hours for the sample which contained no thrombin. These observations were checked using globulin fraction III-3 in place of the fibrinolysin preparation, and the results of these lytic time measurements are given in Table III. It can be seen that the solutions containing the higher concentration of thrombin retained their fibrinolytic activity better than samples with little or no thrombin present.

Summary. It has been shown that fibrinolysin does not affect the clotting activity of thrombin, and that the thrombin preparation increased the stability of fibrinolysin under the conditions of these experiments.

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Effect of Iodine and Adrenalin on Thyrotropin in Graves' Disease and in Normal and Thyroidectomized Dogs.

LOUIS J. SOFFER, MARIO VOLTERRA, J. LESTER GABRILOVE, ABOU POLLACK, AND
MILDRED JACOBS.

From the Department of Medicine and the Laboratories, The Mount Sinai Hospital, New York City.

This study deals with (1) the evaluation of the amount of circulating thyrotropic factor in clinical hyperthyroidism, (2) the effect of iodine on such circulating thyrotropic factor, and (3) the influence of the parenteral injection of adrenalin on thyrotropic discharge from the anterior pituitary in the intact and the totally thyroidectomized experimental animal.

Method. Biological assay was used for the determination of thyrotropic factor. This technic involves histological examination of the thyroids of young guinea pigs not ex-

ceeding 200 g in weight, following subcutaneous injection of 5 cc of serum from the patient or experimental animal. The guinea pigs were injected on 2 successive days and were killed with ether 24 hours after the last injection. The thyroid lobes were then removed, fixed in 10% formalin, and stained with hematoxylin-eosin. The thyroids were then examined for hyperplastic changes. Following the injection of varying amounts of pure thyrotropic factor there occur changes in the thyroid characterized by a decrease in the amount of colloid in the follicles, an in-

crease in the height of the cells lining the acini, a decrease in the size of the alveoli, and not infrequently mitosis may be observed.¹⁻⁴ These findings are not dissimilar to those observed in the thyroids of patients with Graves' disease. The amount of circulating thyrotropic factor in the serum of our patients and experimental animals was determined on the basis of the criteria just outlined.

Specimens of blood were obtained from patients with Graves' disease before treatment and again on the 2nd, 4th, 6th, 8th, and 11th days after lugolization was started. Five cc of the serum was injected into the guinea pigs on 2 successive days. The animals were killed 24 hours later and their thyroids promptly removed. On several patients samples of blood were also obtained on the 2nd, 4th, and 6th days, after subtotal thyroidectomy. The animal experimental studies were conducted in dogs. Normal dogs were injected with 1 cc of adrenalin-in-oil twice daily. On the 4th day of therapy one lobe of the thyroid was removed for histologic study. The injections were continued and the second lobe of the thyroid was removed 10 days later. In another group of animals a control sample of blood was obtained, a total thyroidectomy performed, and injections of 1 cc of adrenalin-in-oil twice daily were then begun. Specimens of blood were obtained on the 2nd, 4th, 6th, 8th, and 10th days after thyroidectomy. Five cc of serum was injected into the guinea pigs on 2 successive days and the guinea pigs' thyroids

were removed 24 hours later.

Results. Thirteen patients with Graves' disease were studied in this fashion. In all these patients prior to treatment there was less circulating thyrotropic factor than is found to be present in normal individuals. This is consistent with the observation previously reported by Rawson and Starr.⁴ Following lugolization there occurred an increase in circulating thyrotropic factor in all patients, which reached a peak between the 4th and 6th days and then began to diminish. In 3 instances the increase in circulating thyrotropic factor was marked, as evidenced by the profound hyperplastic changes observed in the guinea pig thyroids. In the remaining 10 patients the increase in circulating thyrotropic factor varied from slight to moderate. In a control series of 6 normal individuals who were given Lugol's solution and studied in an identical manner there occurred a barely perceptible increase in circulating thyrotropin. After subtotal thyroidectomy in patients with Graves' disease there occurred a further slight increase in the thyrotropic factor in the blood. This confirms the observations of Rawson and Starr,⁴ who noted a similar although much more marked increase after total thyroidectomy.

Following the injection of adrenalin-in-oil in the intact dogs there occurred marked hyperplastic changes in the thyroid lobe removed on the 4th day of injection. The further administration of adrenalin-in-oil resulted in a considerable decrease in the hyperplasia of the remaining lobe which was removed 10 days later.

In the totally thyroidectomized dogs the injection of adrenalin-in-oil resulted in a marked increase in circulating thyrotropic factor, which reached its peak approximately 4 to 6 days after the beginning of treatment and thereafter began to diminish.

¹ Loeb, L. (cited by Rabinovitch, J.), *Am. J. Path.*, 1928, **4**, 601.

² Junkmann, K., and Schoeller, W., *Klin. Wchnschr.*, 1932, **11**, 1176.

³ Heyl, J. G., and Laqueur, E., *Arch. Internat. de Pharmacodyn. et de Thérap.*, 1935, **49**, 338.

⁴ Rawson, R. W., and Starr, P., *Arch. Int. Med.*, 1938, **61**, 726.

Recovery of the Leukocytosis-Promoting Factor in Exudates of Rabbits.*

VALY MENKIN. (With the assistance of Arnold A. Swanson.)

From the Department of Surgical Research, Temple University School of Medicine, Philadelphia, Pa.

Earlier studies have demonstrated the presence of a leukocytosis-promoting factor in inflammatory exudates.¹⁻⁵ This factor, which has been abbreviated as the LPF has been found in canine exudates and human exudates. Canine material has been found to be active in guinea pigs and in human beings, and the factor present in human exudates was found to be active on dogs.

The factor has not been successfully isolated from rabbit exudate although its presence has been demonstrated by the author,⁶ and has been confirmed by Reifenshtein and his collaborators.⁷ Nevertheless attempts to extract the factor from rabbit exudates resulted in failure. The rabbit is well known to be somewhat atypical as to the type of polymorphonuclear cells it contains in exudates, the type of intracellular enzymes, and the changes in the hydrogen ion concentration of the exudate, with the progress of inflammation. In the present communication observations have been collected which indicate that the factor can be extracted from rabbit exudates. Its effect on the absolute white count is not very striking when a study of normal variations is made on rabbits. However, when one studies the types of leukocytes which are being discharged as a result of

the injection of the LPF, it is clear that the leukocytosis-promoting factor operates primarily in inducing a rise in the percentage of immature leukocytes. It is interesting in this connection that Danzer⁸ showed that the injection in rabbits of various extracts of organs deflects the polymorphonuclear count. The extracts were prepared from normal muscle, liver, brain, and testis by shaking the minced tissue in saline. Ponder and MacLeod⁹ demonstrated that the repeated injections of an irritant into the peritoneal cavity is correlated with a showering into the blood stream of very young polymorphonuclear leukocytes of Class I.

The method employed to extract the LPF from rabbit exudates can be outlined as follows:

1. Inflammatory exudates are recovered from the pleural cavity by injection of 0.2 cc of 5% croton oil in olive oil. A day or 2 later variable amounts of exudates are obtained from such region.

2. Such exudates are immediately treated with benzene in a proportion of 1:1.

3. The mixture of benzene and exudate is stirred under a hood with a stirring apparatus for a period of about 20 minutes.

4. The well-mixed contents are poured into a separatory funnel and held in such a container until good separation occurs.

5. The benzene-free fraction is now treated with ammonium sulfate at half saturation.

6. The precipitate is centrifuged. The supernatant fluid is discarded and the precipitate is dialyzed until free of sulphate ions. It is then injected in variable amounts into the marginal vein of a normal rabbit. Counts

* Aided by a grant from the Agnes Barr Chase Foundation for Surgical Research.

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2 Menkin, V., *Arch. Path.*, 1940, **30**, 363.

3 Menkin, V., and Kadish, M. A., *Am. J. Med. Sci.*, 1943, **205**, 363.

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5 Menkin, V., *Arch. Path.*, 1946, **41**, 376.

6 Menkin, V., *Dynamics of Inflammation*, Macmillan Co., New York, 1940.

7 Reifenshtein, G. H., Ferguson, J. H., and Weiskotten, H. G., *Am. J. Path.*, 1941, **17**, 233.

8 Danzer, M., *Quart. J. Exp. Physiol.*, 1930, **20**, 141.

9 Ponder, E., and MacLeod, J., *J. Exp. Med.*, 1938, **67**, 839.

TABLE I.
Effect of Leukocytosis-promoting Factor of Rabbit Exudates on Number of Circulating Leukocytes.

Rabbit No.	Amt of LPF injected, cc	Basal white cell count, per mm ³	Basal No. of immature WBC (one lobe), %	Max. No. of white cells within 6 hr following injection of LPF, per mm ³	Max. No. of immature WBC (one lobe), %
1-53	5	9,325	2	13,950	18
1-73	22	9,600	6	17,500	38
1-59	13	8,875	2	15,400	38
19-77	20	6,850	—	16,300	30
19-73	6	10,975	4	15,400	30
1-78	9	7,600	10	21,600	35
Avg		8,871	4.8	16,692	31.5

TABLE II.
Effect of Saline and Other Inert Material on Number of Circulating Leukocytes in the Rabbit.

Rabbit No.	Amt of material inj. cc	Basal white cell count, per mm ³	Basal No. of immature WBC (one lobe), %	Maximum No. of white cells within 6 hr after injection, per mm ³	Maximum No. of immature WBC (one lobe), %
2-5	5 Saline	8,400	4	14,850	4
19-92	5 "	7,825	—	9,750	—
2-5	5 "	6,350	—	17,400	—
18-24	6 Serum (rabbit)	5,350	0	9,250	2
19-97	15 Saline	6,625	—	11,550	—
1-82	5 Benzene in horse serum	8,100	—	13,650	—
1-88	10 Saline	9,075	4	16,100	14
19-73	20 "	8,225	4	12,150	2
1-61	8 Horse serum	9,325	4	13,400	10
Avg		7,697	3.2	13,122	6.4

are made every hour for several hours and blood smears are likewise taken.

The results of such a series of experiments appear in Table I. It is clear that although the rise in the circulating leukocytes is not excessive, yet the percentage of immature leukocytes is markedly increased as a result of such an injection.

The results obtained when the normal variation in the leukocytes of rabbits is studied indicate that the striking effect of the LPF is primarily on the differential count. The

same fails to hold when various inert materials are injected into the circulating blood of normal rabbits. The results appear in Table II.

Summary. The leukocytosis-promoting factor is present in rabbit exudates, and it has been extracted from such fluids. The extracted LPF has primarily an effect in inducing the discharge of immature leukocytes. The effect on the absolute level of leukocytes is to increase it, but this effect is not pronounced.

Susceptibility of the Gray Fox to Fox Encephalitis.

R. G. GREEN AND C. S. STULBERG.

From the Department of Bacteriology and Immunology, University of Minnesota Medical School, Minneapolis.

Studies on the adaptation of the virus of fox encephalitis to animal species have shown that this virus can invade related groups of animals with varying degrees of pathogenicity. In members of the family Canidae, such as the red and silver foxes, dogs, and coyotes,¹ experimental infection with the virus results in a high mortality, while such species as the black bear² and the raccoon,³ which are offshoots of the canines, are only slightly susceptible.

The gray fox (*Urocyon*) is only distantly related to the red fox group (*Vulpes*). The latter is related to European forms, whereas the gray fox is probably a survivor of American preglacial fauna and appears to be more closely related to the South American foxes and dogs. In view of these relationships, it has been of interest to study the susceptibility of gray foxes to fox encephalitis.

Our earliest experiments¹ indicated that gray foxes inoculated with the virus by cisterna puncture did not show evidence of infection. In a later experiment one of 2 gray foxes inoculated by simultaneous cisterna puncture and intramuscular injections developed symptoms of fox encephalitis and was killed at the end of 4 days. Microscopic examination revealed characteristic inclusion bodies in the meninges and brain capillary endothelium. Inclusions were also seen in hepatic and reticular-endothelial cells of the liver and in the endothelium of the kidney glomeruli. More recently we have been able to show that a symptomless infection can be

produced regularly in gray foxes and that only occasionally will an infection result in death.

Attempts were made to infect gray foxes by intracerebral, intraperitoneal, or intraocular injections. Such inoculations in red foxes result in an acute systemic infection with a high mortality.^{4,5} Endothelial cells of organs infected, particularly the endothelial cells of the brain capillaries, show intranuclear inclusion bodies which are characteristic of the disease.⁶ When the virus is inoculated into the anterior chamber of the eye, the single layer of endothelial cells lining the posterior surface of the cornea is infected. This is indicated by the presence of inclusions and the development of a corneal opacity.⁵

The virus regularly used in our experimental work is a homogenized suspension of infected fox brain preserved as 20% tissue in 50% neutral glycerin. The source of each of virus lots 77, 88, and 98 were pooled brain suspensions from not less than 10 infected red foxes which were allowed to die from the infection or were killed *in extremis*. These pooled viruses were subsequently tested in red foxes and were found to be highly virulent.

In a preliminary experiment the 20% suspension of pooled lots 77, 88, and 98 was diluted to a 2% suspension in Ringer's solution. 0.1 cc was injected into the anterior chamber of the left eye of an 8-weeks-old gray fox pup. In 3 days the eye was completely opaque. The animal was killed, and smears of the corneal endothelium showed

¹ Green, R. G., Ziegler, N. R., Carlson, W. E., Shillinger, J. E., Tyler, S. H., and Dewey, E. T., *Am. J. Hyg.*, 1934, **19**, 343.

² Stulberg, C. S., and Green, R. G., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 88.

³ Green, R. G., Evans, C. A., and Yanamura, H. Y., *Proc. Soc. Exp. Biol. and Med.*, 1943, **53**, 186.

⁴ Green, R. G., Ziegler, N. R., Dewey, E. T., and Shillinger, J. E., *Am. J. Hyg.*, 1931, **14**, 353.

⁵ Evans, C. A., Yanamura, H. Y., and Green, R. G., *Proc. Soc. Exp. Biol. and Med.*, 1943, **53**, 183.

⁶ Barton, J. C., and Green, R. G., *Am. J. Hyg.*, 1943, **37**, 21.

typical intranuclear inclusions. The aqueous humor was bacteriologically sterile. No inclusion bodies were observed in the brain capillaries. Presence of the virus in the brain of the gray fox was shown by aseptically removing a portion of the cerebellum, which was then ground with mortar and pestle and diluted to a 5% suspension with Ringer's solution. 0.1 cc was injected into the anterior chamber of the eye of the highly susceptible red fox, which developed a corneal opacity in 4 days and died of typical fox encephalitis in 5 days.

0.25 cc of 20% pooled virus from lots 77, 88, and 98 was then inoculated intracerebrally into two 8-weeks-old gray fox pups. At the end of 5 days these animals had not developed symptoms, although with red foxes a 2- to 4-day incubation period was commonly observed under similar conditions. The gray foxes were killed and their brains and livers were removed aseptically. Microscopic examination revealed no evidence of infection. Equal parts of the brains were pooled and the mixture diluted to 20% in Ringer's solution. This was designated "gray fox passage virus" and was inoculated into a second group of 4 young gray fox pups as follows: (1) 0.25 cc intracerebrally and 0.1 intraocularly into the anterior chamber of the left eye; (2) 0.25 cc intracerebrally; (3) and (4) 0.25 cc intracerebrally simultaneously with 0.25 cc of pooled 20% virus from lots 77, 88, and 98.

The first gray fox, which was inoculated with gray fox passage virus intracerebrally and intraocularly, developed a slight corneal opacity in 3 days. The opacity became progressively marked by the end of 8 days, at which time the animal appeared sluggish in its movements and would not eat. The fox was killed, and inclusions were found in the corneal endothelium but not in the brain or other tissues. A portion of the brain was removed aseptically, ground and diluted to a 5% suspension in Ringer's solution, and injected into the anterior chamber of the eye of a red fox. This animal developed a positive eye reaction in 3 days and died in 5 days with symptoms and microscopic findings of fox encephalitis. The second gray fox, inoculated with gray fox passage virus intra-

cerebrally, was normal at the end of 5 weeks.

The 3rd and 4th gray foxes were inoculated intracerebrally with gray fox passage virus together with equal amounts of original red fox virus. One animal remained normal, while the other developed symptoms of fox encephalitis by the 8th day. This animal had occasional convulsions and exhibited a marked weakness of its hind legs. When standing up, it appeared to be in a lethargic state, but weaved back and forth unsteadily. The fox was killed *in extremis*. Numerous intranuclear inclusions were found in the brain capillary endothelium and in the liver reticular-endothelium. Material from the brain of this animal, subsequently injected intraocularly into a red fox, produced the typical eye reaction, symptoms, and death, with typical pathology of fox encephalitis.

The 2 gray foxes that remained normal in this group developed a distemper infection 5 weeks later and were discarded.

Pooled brain and liver from all the gray foxes that showed evidence of a fox encephalitis infection, together with the original pooled virulent red fox virus from lots 77, 88, and 98, were inoculated into a group of 4 young gray foxes. 0.5 cc of a 20% suspension of the pooled tissues was injected intracerebrally, simultaneously with 2 cc injected intraperitoneally. Two of the animals were sacrificed at the end of 6 days, although no symptoms had been observed. Microscopic examination of the brains of both animals revealed several typical intranuclear inclusions in the capillary endothelium. The brain material, subsequently inoculated intraocularly into red foxes, produced the characteristic eye reactions, symptoms, and death from fox encephalitis. The 2 gray foxes that were allowed to live remained normal for 5 weeks, after which time they developed a distemper infection and were discarded. When brain from one of these animals was inoculated intraocularly into a red fox, no viable virus was found.

Summary. The results indicate that the gray fox is relatively resistant to fox encephalitis virus virulent for red foxes. It appears that the virus will regularly infect the gray fox to produce a symptomless infection,

but only occasionally will symptoms appear that resemble the disease in red foxes.

A virulence gradient of this virus for related species can be postulated whereby in the family *Canidae* the closely related dogs and coyotes (*Canis*) and red foxes (*Vulpes*)

are most susceptible, the more distantly related gray fox (*Urocyon*) less susceptible, and those species which are earlier offshoots of the canines, such as the black bear and the raccoon, are the least susceptible to fox encephalitis.

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Viability of the Rabbit Papilloma Virus.

R. G. FISCHER AND R. G. GREEN:

From the Department of Bacteriology and Immunology, University of Minnesota Medical School, Minneapolis.

Rabbit papillomatosis was described by Shope,¹ who established that the tumorous growth was due to a filterable virus. The occurrence of the rabbit papilloma seems to have been first recorded by Seton,² but the warty growths generally described as rabbit horns were well known even previously to hunters and naturalists. Thaddeus Surber³ noted the growths on Kansas cottontail rabbits in 1899.

Our first studies of rabbit papillomatosis were in 1927 and since samples of rabbit horns collected by Green at that time were preserved, it is now possible to test samples for viability that have been in storage for almost 20 years. Recently we have tested a group of 6 viruses in storage from approximately 6 to 20 years.

From the reports in the literature, it has become obvious that stored papilloma tissue

would remain viable for long periods of time. Shope in his original work used papilloma tissue which had been stored 106 days. Bryan and Beard⁴ used papilloma tissue which had been stored for 3 years and found it to be capable of producing infections. Samples of the Shope virus in our laboratories have been stored as pieces of papillomatous tissue in 50% glycerine, refrigerated at 4°C.

The tissues were tested for viable virus by scarifying small areas of skin on the ears and flanks of domestic rabbits and applying small amounts of centrifuged tissue suspensions. In preparing material for inoculation, tissues were ground with a mortar and pestle and suspended in 9/10% saline to give a tissue suspension of about 10%. The suspension was centrifuged for one-half hour at 2000 r.p.m. The supernatant was used for the skin inoculation and was applied with a

TABLE I.
Duration of Viability of Shope Papilloma Virus.

Tissue No.	Source of tissue	Date collected	Date tested	Length of proven viability
1	Cottontail rabbit	Mar. 1927	Jan. 1947	19 years 10 mo.
2	" "	Jul. 1937	Nov. 1946	9 " 4 "
3	" "	Apr. 1938	Jun. 1946	8 " 2 "
4	" "	Feb. 1939	Jun. 1946	7 " 4 "
5	" "	Mar. 1939	Jul. 1946	7 " 4 "
6	" "	Apr. 1940	Sep. 1946	6 " 5 "

¹ Shope, R. E., and Hurst, E. W., *J. Exp. Med.*, 1933, 58, 607.

² Seton, E. T., *Lives of Game Animals*, Vol. IV, Part II, 787.

³ Personal note to R. G. Green.

⁴ Bryan, W. R., and Beard, J. W., *J. Nat. Cancer Inst.*, 1941, 1, 607.

cotton swab. The dates of virus collection and testing are given in Table I.

All of the above tissues proved capable of producing typical confluent papillomatous growths in domestic rabbits. The incubation period in each case was approximately 2 weeks except for tissue No. 1, for which the incubation period was about $3\frac{1}{2}$ weeks.

Summary. Cottontail papilloma tissue stored in 50% glycerine at 4°C remains infective and capable of producing confluent papillomatosis for as long as 20 years. All 6 of the tissues tested after storage from 6 to 20 years were found viable. This seems to establish that Shope virus is extremely stable when stored under refrigeration.

15828

A Pithed Rat Preparation Suitable for Assaying Pressor Substances.

R. E. SHIPLEY AND JOHN H. TILDEN.

From the Lilly Laboratory for Clinical Research, Indianapolis City Hospital, Indianapolis, Ind.

Although pithed cats and pithed dogs are very useful as sensitive test preparations for studying or assaying pressor substances, the cost for procurement and maintenance of the animals is a considerable disadvantage. Because rats are more readily available and are much more economical to use when large numbers of test animals are involved, an effort was made to develop a pithed rat preparation which would satisfy the requirements for pressor sensitivity and reproducibility. In the present paper a technic is described for setting up a satisfactory rat preparation which compares favorably with, and in several respects is superior to, the pithed cat or dog.

Young male rats (albino or hooded) weighing 150-300 g were used. The rat was anesthetized by the intraperitoneal injection of sodium amytal, 0.09 mg/g body weight. Atropine sulfate (1.2 mg) was given along with the anesthetic. The trachea was exposed and a piece of plastic tubing or rubber catheter 6 cm long and 2.5 mm in diameter was inserted into the opened trachea. Tubing of this size fitted snugly and did not need to be tied in place.

Either the femoral or common carotid artery was isolated for cannulation. A short cannula of small diameter (0.5 mm) was tied in the artery and connected to a mercury manometer by plastic or rubber tubing. A dilute solution of heparin was used to prevent

clotting in the cannula. (It is advisable to use a small bore [3.0-3.5 mm] mercury manometer to minimize the loss of blood from the rat by displacement into the manometer during rises in blood pressure.)

The vagi were then cut and the jugular veins and carotid arteries tied. With the rat on its back and the hind feet pinned to the operating board the animal was ready to be pithed. The pithing rod, 2.2 mm in diameter, 25 cm long, with one end bluntly pointed, was made from an ordinary wire coat hanger. By holding the rat's head taut and in line with the vertebrae, with the thumb in the angle of the mandible and the forefinger around the top of the skull, the point of the pithing rod was inserted obliquely into and through the eye socket at an angle of approximately 45° to the long axis of the rat. After the skull was entered the rod was realigned with the vertebral column and passed through the cranium and thence down the whole length of the spinal canal. The pithing rod was left in place on the assumption that it would afford mechanical compression of blood vessels torn by the rod in the process of pithing.

The tracheal cannula tubing was then promptly connected to the respirator system which included a side tube fitted with an adjustable screw clamp. The respirator used was an air-driven, windshield wiper type with adjustments for varying both rate and

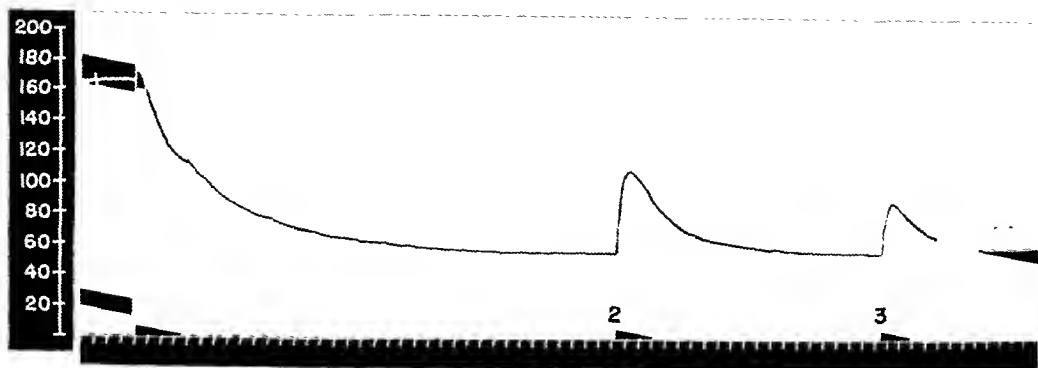


FIG. 1.

Record of mean blood pressure in a pithed rat preparation. Wt. 180 g. 1. Rat pithed. 2. I.V. injection of 6 μ g of a "laboratory standard" angiotonin powder in 0.2 ml saline. 3. I.V. injection of 0.1 μ g epinephrine hydrochloride in 0.2 ml saline. Ordinate scale—blood pressure in mm Hg. Time marker—1 minute. (32ZD-14.)

volume. (Great care must be taken to avoid hyperventilation or overdistention of the lungs if the development of pulmonary edema is to be prevented.) Respiratory rates of 50-60 per min. were found to be optimum for the rat. Slower rates of 30-40 were tolerated but oxygenation of the blood appeared to be inadequate. With rates slower than 20 the rats lived for only a short time.

For making intravenous injections a small portion of skin was cut away from the area overlying the femoral vein. Into one end of a 6-inch piece of very small plastic tubing (1 mm O.D.)* was fitted the shaft of a 24-gauge hypodermic needle which had been cut off below the syringe hub. The needle was inserted into the femoral vein and left in place, the application of a ligature being unnecessary. Syringes of 0.25 or 1.0 ml capacity and graduated in 0.01 ml were fitted with 26- or 24-gauge hypodermic needles the beveled points of which had been ground off flat. Usually 0.1 to 0.2 ml of test sample was given, syringe and needle removed from the plastic tube and replaced by a second syringe and needle with 0.1 ml physiological saline wash. Care had to be taken to avoid the inclusion of very small air bubbles when changing syringes. This could be accomplished by injecting 0.02 ml of saline and quickly replacing syringe and needle with the

test sample syringe and needle. During this exchange a slight outward flow of saline would expel the small air bubble usually present as the result of the transfer.

The animal was left undisturbed for $\frac{1}{2}$ hour before injections were begun during which time the depth of respiration was observed and altered if necessary according to the color of the arterial blood and the extent of movement of the thorax. With a little practice it was found that the optimum exchange of air could be gauged with reasonable accuracy from the excursions of the chest wall.

Thirty to 40 minutes after the rat was pithed the mean blood pressure usually leveled off between 40 and 60 mm Hg. Pressor responses of 30-40 mm Hg were obtained in most of the preparations with only 0.05-0.1 μ g of epinephrine. Occasionally comparable rises in blood pressure were obtained with as little as 0.0125 μ g. In general, the sensitivity to angiotonin was from 2 to 5 times as great as that exhibited by the pithed cat. A sample record of typical pressure curves is shown in Fig. 1.

With proper maintenance of the depth and rate of artificial respiration, satisfactory pressor responses were obtained for periods averaging 5 to 7 hours. Over 120 pithed rats have been prepared by the method described and approximately 90% of these have been satisfactory test animals. In the ma-

* "V-1" plastic tubing, A. C. Balfour Associates, Englewood, N.J.

jority of instances technical errors or the use of rats which showed signs of illness were thought to be responsible for preparations which exhibited poor pressor responses or died prematurely.

Summary. A simple method is described for setting up a sensitive pithed rat prepara-

tion suitable for assaying small quantities of pressor substances.

Grateful acknowledgment is made to Mr. C. Wilson whose technical experience in preparing pithed cats has been a valuable aid in the present study.

15829

Effect of Pentaquine in Patients with Hypertension.*

EDWARD D. FREIS AND ROBERT W. WILKINS.

From the Evans Memorial, Massachusetts Memorial Hospitals, and the Department of Medicine, Boston University School of Medicine.

The recent observation that pentaquine [6-methoxy-8-(5-isopropylaminoamylamino)-quinoline], a new antimalarial agent, produces postural hypotension in normal man^{1,4} raised the question of its usefulness in treating hypertensive patients. The purpose of the present report is to summarize briefly the results of a clinical trial of this compound in a random group of 17 patients with long standing essential hypertension including 3 in the malignant phase of the disease.

Procedure and Results. Pentaquine was administered orally in amounts varying from 120 to 240 mg of the base per day, given in equally divided doses every 4 hours. Arterial pressure was measured in the arm by the standard auscultatory method using a mercury manometer. Measurements were made with the patient resting comfortably in the supine position, and also, during and after 5 minutes (or less if syncope intervened) of motionless standing. Mean arterial pressure was calculated as one-half the sum of

the systolic and diastolic blood pressure. The pulse rate was palpated at the wrist or auscultated at the cardiac apex. Patients were hospitalized for at least 3 days prior to treatment, and were maintained on essentially the same regimen before, during and after the period of drug therapy.

In the majority of cases (Table I), after 2 to 7 days of treatment at dosages of 120 to 240 mg per day, a reduction of systolic and diastolic blood pressure occurred, varying from 10 to 40% of the mean arterial blood pressure. At the same time there was usually a further fall in blood pressure in the erect position associated with narrowing of pulse pressure. The pulse rate remained unchanged, or actually decreased in the supine position, and in the majority of patients failed to rise abnormally in the upright position even during marked hypotension.

The development of postural hypotension was not observed in all cases. Four patients exhibited a definite fall in resting blood pressure without the development of a further hypotension when erect. Others maintained a lower resting blood pressure for several weeks after the disappearance of the postural effect. However, one patient exhibited a reduction of blood pressure only in the upright position. In a few patients the depression of arterial pressure first appeared several days after the medication had been discontinued because of toxic reactions.

* This investigation was supported in part by the Squibb Institute for Medical Research, New Brunswick, N.J. The pentaquine used was supplied by James A. Shannon, M.D., of the Squibb Institute.

¹ Loeb, R. F., *J. A. M. A.*, 1946, 132, 321.

⁴ Craigie, B., Jr., Jones, R., Eichelberger, L., Alving, A., Pullman, R. N., and Whorton, C. M., to be published.

TABLE I.
Summary of Cases.

Sex	Age	Blood pressure prior to treatment	Dosage pentaquine, mg/day	Supine blood pressure at time of max. fall	Days to return to pretreatment blood pressure	Postural hypotension	Toxic effects
F.	50	220/120	120-2 days	150/90	12+	+++	++++
M.	41	210/130	180-3 "	180/118	2	+	+++
F.	36	240/140	240-1 "				
F.	48	180/110	120-3 "	Discontinued	Severe vomiting and pain		
			120-3 "	140/90	3+	+	++
M.	46	200/100	180-4 "				
			180-2 "	No response			++
			240-2 "				
F.	46	230/130	120-2 "	125/88	6	++++	+++
			180-3 "				
F.	41	230/130	120-3 "	150/100	20	++	++
			180-3 "				
F.	41	220/120	120-14 "	No response			
F.	46	170/110	120-2 "	140/90	20+	++	+
M.	34	170/120	120-2 "	135/110	7+	0	++
			180-4 "				
			240-4 "				
F.	46	220/140	120-4 "	180/120	4	0	++
			60-6 "				
F.	49	240/150	120-3 "	240/150	Postural hypotension 5 days		+
			180-4 "				
M.	51	200/130	120-3 "	170/90	30+	0	+
			90-7 "				
F.	49	260/135	120-2 "	190/100	15	0	++
			180-4 "				
			60-11 "				
M.	47	250/170	120-3 "	140/70	Terminal uremia		
F.	34	240/140	90-11 "	140/90	20	+++	++
			120-6 "				
M.	45	230/160	120-2 "	160/100	12	+++	+++

The last 3 patients were in the malignant phase of essential hypertension.

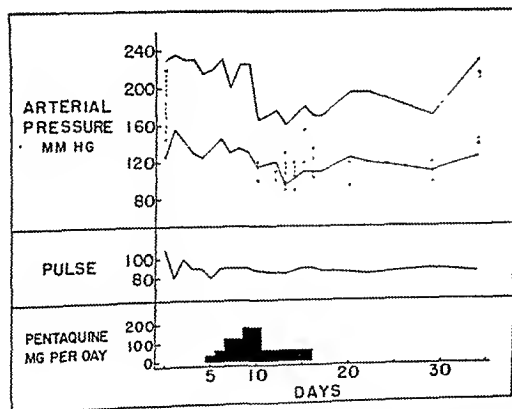


FIG. 1.

Typical response to pentaquine. The vertical broken lines represent arterial pressure readings after 2 minutes in the upright position.

The effective dose varied markedly, one patient requiring 120 mg per day given for

2 days, while several failed to respond to doses as high as 240 mg given for 5 days. Fig. 1 illustrates the typical response of a patient with essential hypertension.

The lowering of blood pressure was often preceded and accompanied by abdominal pain and tenderness, back and chest pain, frequently girdle in character, facial pallor, anorexia, nausea and vomiting, constipation or diarrhea, loss of weight in a few patients, and rarely by fever. Nausea and vomiting and/or abdominal pain were occasionally sufficiently severe to necessitate discontinuation of treatment. Impotence was noted in 2 male patients. Methemoglobinemia and moderate hemolytic anemia occurred in earlier cases but were later successfully controlled by the simultaneous administration of 1 grain of methylene blue with each dose of pentaquine. Cyanosis without signs of cardiac

failure or significant methemoglobinemia appeared in a few patients.

In addition to these toxic effects, agranulocytosis developed in one patient after 2 weeks of continuous therapy, and prolonged menses appeared in another, accompanied by evidence of increased capillary fragility. Both patients recovered uneventfully following cessation of therapy and treatment with penicillin in the case of agranulocytosis. There were no other severe reactions in the group.

Following cessation of full doses of the drug the blood pressure gradually returned to its previous level over a period of several days to several weeks. In most cases the hypotensive effect was not prolonged by the administration of 50 to 60 mg of pentaquine per day. The depression of blood pressure and postural hypotension recurred, however, when the drug was again administered in full doses.

The patients with a malignant phase of essential hypertension as manifested by neuroretinitis, impairment of renal function, high diastolic blood pressure, weight loss and other signs of rapidly progressing disease, responded to pentaquine similarly as patients with uncomplicated essential hypertension, except that they uniformly required lower dosage (120 mg per day). Coincident with the fall in blood pressure there was considerable regression of the pathological changes in the fundi, relief of headaches and cessation of gross hematuria, but apparently no improvement in renal function as measured by clinical and clearance tests.

Hemodynamic studies using Hamilton manometers for recording arterial pressure and the ballistocardiograph for measuring cardiac output, before and after the hypotensive effect had been achieved, indicated that pentaquine caused a reduction of sympathetic vasopressor reflexes similar to that occurring after surgical sympathectomy.² This lack of responsiveness was most apparent in those cases which exhibited marked postural hypotension. Measurements of the skin temperature in the extremities under control con-

ditions also indicated a depression of sympathetic activity.³ Pressor responses to epinephrine and ephedrine were unimpaired, but epinephrine did not prevent collapse in the erect position. Marked postural hypotension and collapse were prevented, however, by the use of a tight abdominal belt similar to that worn following lumbodorsal splanchnicectomy. There were no consistent changes in either the electrocardiogram or in the cardiac output.

Discussion. The most interesting effect obtained with pentaquine in hypertensive patients resembled that found in certain normal subjects,⁴ namely the appearance of postural hypotension. In addition, hypertensive patients often exhibited a reduction in resting blood pressure. The frequent occurrence of toxic symptoms at first suggested that the hypotensive effect was merely a reflection of toxic debility. However, there was no uniform relation between the appearance of other toxic symptoms and of the hypotensive effect. Furthermore, the hypotension was frequently maintained for several weeks after the disappearance of all other toxic symptoms, and in such instances was associated with considerable subjective improvement. These results indicate that while the drug is too toxic in the dosage necessary to produce a fall in blood pressure, its toxic and hypotensive qualities may not be inseparable.

The preliminary studies suggested that the mode of action of pentaquine upon the vasomotor system is to depress sympathetic nervous reflexes. Its activity differed from that of certain other sympatholytic agents such as the tetraethylammonium salts in that the depression of blood pressure was more prolonged and was not accompanied by a marked quickening of pulse rate. This maintenance of a normal heart rate indicates that its depressor effects include the cardiac accelerator mechanisms. That pentaquine did not block the reactivity of the vascular system to pressor agents was shown by the continued effectiveness of epinephrine and ephed-

² Wilkins, R. W., and Culbertson, J. W., unpublished data.

³ Mixer, G., Jr., and Freis, E. D., unpublished data.

drine. It is of interest that Moe and Seevers⁵ reported central impairment of sympathetic reflexes in dogs treated with plasmochin, a closely related 8-aminoquinoline.

Summary. 1. The administration of pentaquine in high dosage to patients with essential hypertension frequently produced a significant reduction in resting arterial blood pressure, usually accompanied by the development of postural hypotension. This depressor effect occurred abruptly after several days of therapy and receded gradually over

a period of several days to several weeks following cessation of therapy.

2. Patients with malignant hypertension exhibited a similar response, but did not require as high dosage. With the fall in blood pressure there was some regression of neuroretinitis, headache and gross hematuria; but no significant improvement in renal function.

3. Pentaquine appeared to exert its effects primarily through a sympatholytic action.

4. Toxic reactions to the drug were too frequent and severe to consider its use practicable in the treatment of essential hypertension.

⁵ Moe, G. K., and Seevers, M. H., *Fed. Proc.*, 1946, **5**, 193.

15830

Hemagglutination with Newcastle Disease Virus (NDV).

ALFRED L. FLORMAN.* (Introduced by F. L. Horsfall, Jr.)

From the Hospital of The Rockefeller Institute for Medical Research.

Newcastle disease of fowl, an acute infection associated with severe intestinal, respiratory and nervous symptoms, was first recognized as a distinct entity by Kraneveld¹ in the Dutch East Indies and by Doyle² in Newcastle, England. Doyle gave the disease its present name and demonstrated that the causative agent is filterable.

Recently there has been increased interest in the virus which causes Newcastle disease, especially because of its capacity to cause hemagglutination.³ In addition, although the western hemisphere was thought to be free of the disease, Beach⁴ showed that there is an immunological similarity between classical Newcastle disease virus (NDV) and the virus of avian pneumoencephalitis, a disease which

is of frequent occurrence in this country. The potential human pathogenicity of the virus for man was indicated in 1943 with the report of an accidental laboratory infection.⁵ This resulted in a brief, mild illness associated with conjunctivitis and general symptoms. In 1946 2 similar laboratory infections⁶ and a small epidemic of conjunctivitis among women who had been exposed to infected fowls were reported.⁷

Burnet³ has raised the possibility of "an affinity between NDV and the influenza virus group" chiefly on the basis of hemagglutinating capacity, infectivity for the chick embryo and apparent particle size. However, no immunological relationship could be demonstrated, and recently Bang⁸ and others⁹ have shown by electron microscopy that NDV

* Aided by a grant from the Welt Fund of Mt. Sinai Hospital, New York.

¹ Kraneveld, F. C., *Nederl. Indisch. Blad. Diergeneesk.*, 1926, **38**, 448.

² Doyle, T. M., *J. Comp. Path. and Therap.*, 1927, **40**, 144.

³ Burnet, F. M., *Austral. J. Exp. Biol. and Med. Sci.*, 1942, **20**, 81.

⁴ Beach, J. R., *Science*, 1944, **100**, 361.

⁵ Burnet, F. M., *Med. J. Austral.*, 1943, **2**, 313.

⁶ Anderson, S. G., *Med. J. Austral.*, 1946, **1**, 371.

⁷ Yatani, J., *J. A. M. A.*, 1946, **132**, 169.

⁸ Bang, F. B., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 5.

⁹ Cunha, R., Weil, M. L., Beard, D., Taylor, A. R., Sharp, D. G., and Beard, J. W., *J. Immunol.*, 1947, **55**, 69.

TABLE I.
Successive Titrations of Newcastle Disease Virus at Room Temperature.*

No. of days chicken red cells were stored †	Dilution of virus‡												End point
	4	8	16	32	64	128	256	512	1024	2048	4096	8192	
1	1	2	2	2	2	2	2	2	2	0	0	0	1024
2	1	2	2	3	3	3	3	3	3	2	±	0	2048
3	1	1	2	1	1	2	3	2	2	2	1	0	2048
4	±	1	2	3	3	3	3	3	3	2	1	0	2048

* Room temperature = 22°C to 27°C.

† Red cells obtained from one chicken, and stored at 4°C, concentration = 0.75%.

‡ Reciprocal of final dilution of pool 3.

Results were read at 30 minutes.

TABLE II.
Successive Titrations of Newcastle Disease Virus at 4°C.

No. of days chicken red cells were stored*	Dilution of virus†											End point
	4	8	16	32	64	128	256	512	1024	2048	4096	
1	4	4	4	4	4	4	3	2	1	±	0	512
2	4	4	4	4	4	4	4	4	2	1	0	1024
3	4	4	4	4	4	4	3	2	1	0	0	512
4	4	4	4	4	4	4	4	3	2	±	0	1024

* Red cells, identical to Table I.

† Reciprocal of final dilution of pool 3.

Results were read at 60 minutes.

is strikingly different from the influenza viruses.

In a study of the phenomenon of hemagglutination, NDV was investigated. It was found that the methods which have been developed for influenza viruses were not satisfactory with NDV. When hemagglutination titrations were carried out at room temperature, there was much less marked agglutination with low virus dilutions than with higher ones. Even with more dilute chicken red cell suspensions and a shorter reaction time as recommended by Brandly *et al.*¹⁰ this disturbing effect persisted and the reactions were difficult to read and to reproduce. It is the purpose of this communication to show how this effect can be circumvented by carrying out hemagglutination reactions with NDV in the cold and to offer an explanation for this peculiar property of the virus. In addition, some of the quantitative aspects of hemagglutination by NDV will be presented.

Virus. A strain of Newcastle disease virus (NDV) was obtained from Dr. G. K. Hirst

who had received it from Dr. F. R. Beaudette. Further passages were carried out by inoculation into the allantoic fluid of 9-12-day-old chick embryos. After approximately 48 hours incubation at 37°C, the eggs were refrigerated at 4°C for 3-18 hours, after which the allantoic fluid was collected. Infected fluid was stored either at -70°C or at 4°C.

Sera. An authentic antiNDV serum was obtained from the Bureau of Animal Industry, U. S. Department of Agriculture, Washington, D. C. Specific antisera were prepared in rabbits by slowly injecting into the ear vein 10 cc of allantoic fluid containing the virus. A second intravenous injection, identical to the first, was given after 6 weeks and serum was obtained 2 weeks later.

Saline. An 0.85% solution of sodium chloride containing 0.01M phosphate buffer at pH 7.2 was used throughout.

Chicken red cells. Blood was obtained from the wing vein of individual chickens in an excess of 2% sodium citrate. The cells were washed twice in the usual manner, and suspensions of desired concentration were prepared in saline from cells which had been packed by 10 minutes centrifugation at 1500 r.p.m.

¹⁰ Brandly, C. A., Moses, H. E., Jungherr, E. L., and Jones, E. E., *Am. J. Vet. Research*, 1946, 7, 289.

TABLE III.
Effect of Temperature on Titrations with Newcastle Disease and Influenza Viruses.

Temp., °C	RBC 1.5%	Virus	Dilution of serum†									End point
			8	16	32	64	128	256	512	1024	2048	
22	chicken	NDV	0	±	1	1	2	3	3	1	0	512
4	"	"	4	4	4	3	3	2	±	0	0	256
22	"	PR8	4	4	4	4	4	4	4	2	1	1024
4	"	"	4	4	4	4	4	4	3	3	0	1024
22	"	Lee	4	4	4	4	4	4	3	1	±	512
4	"	"	4	4	4	4	4	4	3	±	0	512
22	human	NDV	1	2	2	3	3	3	2	0	0	512
4	"	"	2	1	±	0	0	0	0	0	0	8
22	"	PR8	4	4	3	3	3	2	2	1	0	512
4	"	"	4	4	3	3	2	2	2	0	0	512
22	"	Lee	4	4	3	3	3	3	1	0	0	256
4	"	"	4	4	3	3	2	0	0	0	0	128

* Reciprocal of final dilution.

Results were read at 60 minutes.

Test tubes. Pyrex tubes 75 by 10 mm were found to be satisfactory.

Titration of virus. As was stated above, when a titration of NDV is carried out by the hemagglutination technic at room temperature, an effect which simulates a serological prozone appears. This is evident from the results shown in Table I. However, if the reaction is carried out in cold (4°C), as originally suggested by Burnet,³ this effect disappears and clear cut end-points are obtained, as can be seen from the results shown in Table II. Therefore, the following titration technic was employed: Serial 2-fold dilutions of infected allantoic fluid are made in cold saline. To each 0.4 cc of dilution an equal volume of chilled 1.5% chicken red cell suspension is added. The tubes are shaken and immediately placed in the refrigerator (4°C) for one hour. The patterns of sedimented cells are then noted while the tubes are still cold. Complete agglutination is considered to have occurred if a solid plaque of cells, which does not stream on tilting the tube, is seen. It is recorded as 4. Partial agglutination is represented by an even thin deposit of cells on the bowl of the tube, 3; a similar cell pattern with a small central mass of unagglutinated cells, 2; and by a larger central mass with only a few clumps of agglutinated cells along its edge, 1. The end-point is taken as the highest dilution in which a 2 reaction occurs.

When virus titrations are carried out in the cold, the end-point obtained with NDV

is usually lower by 2 to 4 times than when the test is performed at room temperature. (Compare the results in Tables I and II). However, the pattern of hemagglutination and titration end-points obtained in the cold is sufficiently reproducible to permit a reasonably accurate estimate of the dilution which corresponds to one agglutinating unit (*i.e.*, the highest dilution giving a 2 reaction). This estimate is of importance in agglutination-inhibition tests for antibodies against the virus.

Care in the selection of a source of red cells for hemagglutination tests with NDV is important. Although human Group O erythrocytes are agglutinated by NDV, they give much poorer reactions in the cold than at room temperature. Results similar to those shown in Table III were obtained with red cells from each of 3 persons; the end-points were approximately 64 times lower at 4°C than at 22-27°C. Erythrocytes from occasional individual chickens are also markedly insensitive to agglutination by the virus in the cold. By selection, however, chickens can be obtained, the red cells from which give end-points not more than 2 times lower at 4°C than at room temperature. Erythrocytes from such chickens are employed as a routine. Chicken red cell suspensions are stored at 4°C and may be used for at least 4 days without appreciably changing the results of titrations, as is shown in Table II.

The weak agglutination seen with low dilu-

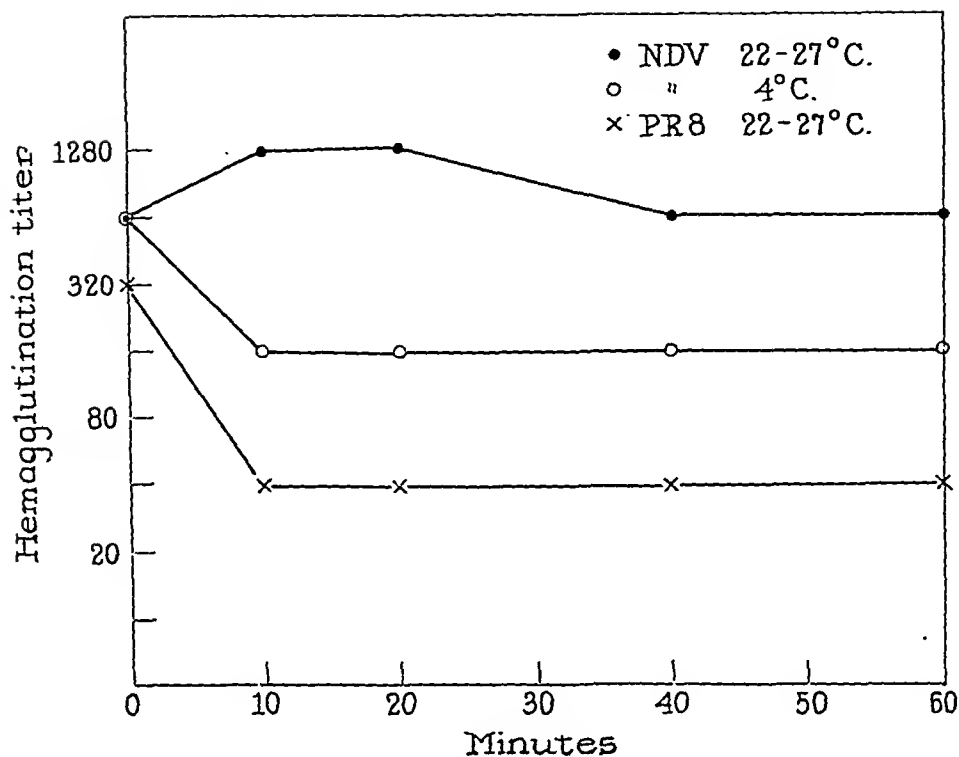


Fig. 1.

Adsorption of NDV and PR8 by chicken red cells. Hemagglutination titers of supernatant at various intervals after mixing red cells and virus.

tions of NDV at room temperature as compared to the complete agglutination observed at 4°C suggested that the difference might be dependent upon the rates of adsorption and elution of NDV from red cells at these temperatures. Experiments were carried out to determine this at 4°C and at room temperature. Equal volumes of 3% chicken red cell suspensions and NDV-infected allantoic fluid were mixed. At intervals aliquots were removed and immediately centrifuged. The supernates were tested for the presence of residual virus by the hemagglutination method. For comparison a similar experiment was performed with influenza A virus at room temperature. The results of these experiments are presented graphically in Fig. 1. Although no evidence was obtained that adsorption of NDV by red cells occurred at room temperature, it seems probable that it actually occurs but is masked because of very rapid elution of the virus from erythrocytes. Adsorption of NDV by

red cells can be demonstrated when the mixture is held at 4°C and at that temperature elution of NDV from erythrocytes does not occur for at least 60 minutes. Hirst¹¹ has shown that temperature influences the rate of elution of influenza viruses from erythrocytes in a similar manner. It appears probable that the difference noted when NDV is titrated at room temperature and at 4°C is dependent on the elution of virus which occurs at room temperature³ with the result that agglutination is masked in lower dilutions of virus. However, Cunha and his associates⁹ have recently reported the presence in allantoic fluid of a substance which interferes with red cell agglutination by NDV.

Hemagglutination caused by NDV may be simply and readily differentiated from that produced by influenza viruses on the basis of the weak agglutination which is obtained with both chicken and human red cells at

¹¹ Hirst, G. K., *J. Exp. Med.*, 1942, **76**, 195.

TABLE IV.
Agglutination-inhibition Tests with NDV and Antiserum.

Preliminary incubation of serum-virus mixtures*	Rabbit serum	Dilution of serum†									End point
		20	40	80	160	320	640	1280	2560	5120	
None	Normal	3	2	3	4	4	4	4	4	4	1:480
	Anti-NDV	±	0	±	0	1	4	4	4	4	
1 hr at 4°C	Normal	4	4	4	4	4	4	4	4	4	1:640
	Anti-NDV	0	0	0	0	0	2	4	4	4	
1 " " 37°C	Normal	4	4	4	4	4	4	4	4	4	1:1920
	Anti-NDV	0	0	0	0	0	0	0	4	4	

* Virus sufficient to yield 8 agglutinating units in the final mixture was employed.

† Reciprocal of final serum dilution.

Results were read after 60 minutes at 4°C.

room temperature with low dilutions of NDV, as well as by the relative failure of human red cells to show agglutination with NDV in the cold. These differences are clearly evident in the experiments summarized in Table III in which parallel titrations of NDV, PR8 and Lee viruses were made with the same suspensions of chicken and human red cells. Allantoic fluid infected with NDV usually gives hemagglutination titers ranging from 1:320 to 1:512, whereas 50% infectivity end-points as determined in chick embryos are of the order of 10^{-10} . This indicates a difference between hemagglutination and virus titers with NDV which is 10 to 100 times greater than that usually obtained with influenza viruses.¹²⁻¹⁴

Titration of antibody. Because of the distinct advantages of performing hemagglutination tests with NDV in the cold, the usual method for measuring antibodies against the virus by means of agglutination-inhibition was modified as follows: The virus is titrated in the cold with the same chicken red cell suspension that is to be used in the agglutination-inhibition test. The final dilution of allantoic fluid which is equivalent to 32 agglutinating units is determined, prepared, and its actual titer checked by a second hemagglutination test. The sera to be tested are

heated at 56°C for 30 minutes and diluted in 2-fold series. To each tube containing 0.2 cc of serum dilution, 0.2 cc of virus (diluted so as to equal 32 units) is added. The mixtures are shaken and incubated for 60 minutes in a water bath at 37°C. They are then cooled to 4°C and 0.4 cc of cold 1.5% chicken red cell suspension is added to each tube. After 60 minutes at 4°C the tests are read and the lowest serum dilution showing 2 plus, or greater, agglutination is taken as the end-point.

The results of parallel agglutination-inhibition tests with antiNDV serum are presented in Table IV. The conditions of preliminary incubation of the serum-virus mixtures were varied as indicated and the advantage of incubation at 37°C for one hour before the addition of red cells is clearly demonstrated. This modification was originally suggested by Burnet.⁵

To determine the reproducibility of serum dilution end-points (*i.e.* antibody titers) obtained by means of the agglutination-inhibition method described above, the following experiment was performed: Sera from each of 4 rabbits which had been immunized against NDV were tested separately against 3 different amounts of virus (*i.e.*, 32, 16 and 8 units, respectively). The results are shown graphically in Fig. 2. The end-points obtained were closely similar with each of the 4 immune sera and a constant amount of virus. Moreover, as the quantity of virus used was increased, the serum dilution end-point decreased proportionately. These results indicate that the reaction between NDV and specific antibody *in vitro* shows a quantita-

¹² Taylor, A. R., Sharp, D. G., Beard, D., Beard, J. W., Dingle, J. H., and Feller, A. E., *J. Immunol.*, 1943, **47**, 261.

¹³ Sharp, D. G., Taylor, A. R., McLean, I. W., Jr., Beard, D., Beard, J. W., Feller, A. E., and Dingle, J. H., *J. Immunol.*, 1944, **48**, 129.

¹⁴ Friedewald, W. F., and Pickels, E. G., *J. Exp. Med.*, 1944, **79**, 301.

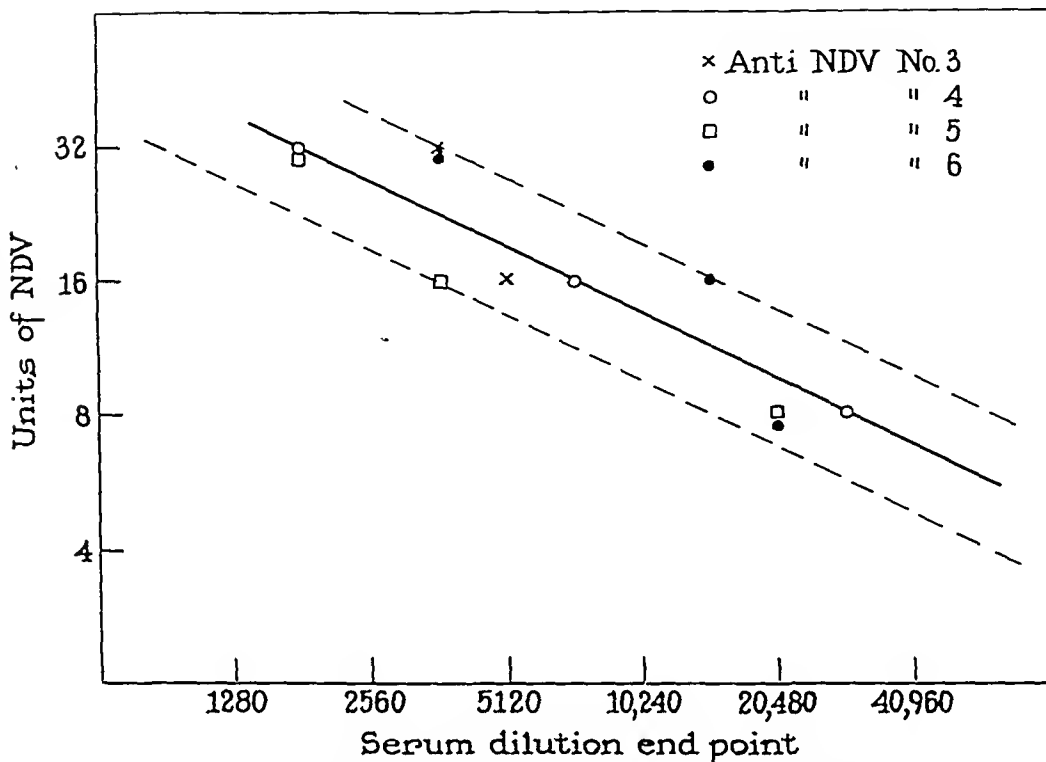


FIG. 2.

Distribution of serum dilution end points in agglutination-inhibition tests with varying amounts of NDV. Antisera from 4 rabbits immunized against NDV. Dotted lines represent the limits of twofold deviations (a single tube) from the solid line.

tive relationship analogous to that previously demonstrated with certain other viruses [*i.e.*, influenza and pneumonia virus of mice (PVM)] as well as with classical antigen-antibody reactions.

Summary. Hemagglutination by Newcastle disease virus (NDV) is markedly affected by temperature. At room temperature the reaction is difficult to read, especial-

ly with low dilutions of virus. At 4°C the reaction is easily read and titration end-points are readily determined.

At 4°C NDV appears to be adsorbed more completely by and to elute less rapidly from chicken red cells than at room temperature.

Details of satisfactory methods for titration of virus and antibodies against it are presented. Both tests are carried out at 4°C.

Effect of Ergotamine Tartrate on Potassium Changes in Histamine Shock.*

CAROLINE TUM SUDEN. (Introduced by H. O. Haterius.)

From the Department of Physiology, Boston University School of Medicine.

Serum potassium changes in histamine shock were investigated in rats treated with ergotamine tartrate (ET-rat) and compared with the previous findings on intact rats and rats with autoplasmic grafts of adreno-cortical tissue (ACT-rat)¹ as an approach to the question of the relative importance of medullary and adrenocortical factors concerned with the release of potassium in histamine shock.

Methods. Serum potassium was determined for adult male rats (Long-Evans strain) at 30 and 60 minutes following intraperitoneal injections of histamine phosphate[†] (10 mg/100 g body weight). Parenteral administration of ergotamine tartrate[‡] (0.5 mg/100 g body weight) was given one-half hour prior to the injections of histamine. The dosage of ergotamine used was sufficient to depress adrenergic activity, as determined by epinephrine reversal, for the strain used. Details of operative procedure and chemical methods of analysis were identical with those previously described.¹

Results. Experiments to date have led to the following observations on the rat treated with ergotamine:

1. Some reduction of the normal fluctuation of serum potassium was indicated by the decrease in the standard deviation.

6 cases, average 21.3 ± 0.8 mg/100 ml
cf. controls 21.3 ± 1.3 mg/100 ml

2. No significant effect upon the rising trend of serum potassium during development of histamine shock (30-minute values) was obtained.

* This investigation has been made with the assistance of a grant from the Committee on Therapeutic Research, Council on Pharmacy and Chemistry, American Medical Association.

¹ Tum-Suden, C., Wyman, L. C., and Derow, M. A., *Am. J. Physiol.*, 1945, 144, 102.

[†] Burroughs Wellcome and Company.

[‡] Gynergen, kindly supplied by Sandoz Chemical Works, Inc.

5 cases, average 24.0 ± 1.9 mg/100 ml
cf. controls, 24.5 ± 1.7 mg/100 ml

3. A possible accentuation in fall of serum potassium occurred during subsidence of histamine shock (60-minute values).

5 cases, average 19.8 ± 2.5 mg/100 ml
cf. controls 22.8 ± 3.5 mg/100 ml

4. A possible exacerbation of the rise in serum potassium ensued upon repeated or extensive bleeding (see 6, below).

3 cases, average 27.9 ± 0.26 mg/100 ml
cf. controls 23.8 ± 1.4 mg/100 ml

The determinations were made on a second blood specimen of 4 ml taken immediately after the first 4 ml bleeding.

5. An elevation of serum potassium followed upon higher dosages of histamine. After 20 and 40 mg histamine in single paired experiments the values obtained 60 minutes later were respectively:

26.8 mg/100 ml and 30.6 mg/100 ml for the ET-rats;

cf. 24.4 mg/100 ml and 24.0 mg/100 ml for controls.

6. No marked influence on gross symptoms was found. Atonia appeared slightly aggravated; under anesthesia respiratory failure upon bleeding was more easily induced than in the controls. The higher values for serum potassium as found in (4) and (5) above correlated with this observation. Toxic reactions from ergotamine alone were not especially apparent in the rat and were in no way as severe as reported for the dog (2).

Some observations were made on rats bearing grafts of adrenocortical tissue and similarly treated with ergotamine. Blood samples were analyzed for potassium at 30 minutes (3 cases) and at 60 minutes (4 cases) following the injection of histamine (10 mg/100 g). The potassium averages for the 2 groups were comparable to those obtained in histamine-shocked rats with only regenerated cortex: *e.g.*

35.5±0.5 mg/100 ml at 30 min., ergotamine;
 34.2±5.8 " " " " " , no ergotamine;
 41.0±2.4 " " " " 60 " , ergotamine;
 38.5±6.5 " " " " " , no ergotamine;

The ergotamine-treated preparations differed from the nontreated in showing less variability in potassium levels. Survival, as judged both by time and number, seemed slightly better than expected for this class of animal receiving a dosage of histamine (10 mg) which previously was fatal to 60% of the nontreated within one hour.

Discussion. The data obtained indicated that fluctuations in serum K tended to be suppressed by ergotamine tartrate. In the dosage used the difference from the untreated controls was not significant. However, if maximum amounts of ergotamine had been employed a slight lowering of the potassium levels might have been established as found by Houssay for the dog.²

Symptomatically, the course of histamine shock following upon the administration of

10 mg intraperitoneally appeared unchanged both in the intact rat and the rat bearing adrenocortical grafts. There was no evidence of a potentiation or synergism between cortical hormones and epinephrine as might be surmised from the findings of Vogt that the latter prolongs cortin secretion.³ The lack of such interaction therefore cannot account to any extent for the high susceptibility in rats possessing only adrenocortical tissue. The experiments serve to emphasize the inefficiency of regenerated cortical substance, the relation of the rise in extracellular potassium to level of cortical activity and the minor influence of the sympathico-adrenal mechanisms upon potassium release in histamine shock.

Summary. Ergotamine tartrate in the dosage used did not significantly modify serum potassium changes characteristic of histamine shock in the rat. It did not influence histamine tolerance in either the normal or the demedullated animal.

² Houssay, B. A., and Gerschmann, R., *Rev. Soc. Argent. de Biol.*, 1939, 15, 327.

³ Vogt, M. J., *J. Physiol.*, 1945, 103, 319.

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Effect of Sex Hormones on Pituitary Lactogen and Crop Glands of Common Pigeons.*

JOSEPH MEITES AND C. W. TURNER.

From the Department of Dairy Husbandry, University of Missouri, Columbia, Mo.

Estrogens, testosterone propionate and progesterone can exert a dynamic stimulus to the secretion of lactogenic hormone by the anterior pituitary. This is particularly true of the estrogens which have been shown to increase the pituitary lactogen content of

rats,¹⁻³ guinea pigs^{3,4} and rabbits⁵ from 200 to 500%. A 40% increase has been obtained with testosterone propionate in rats.⁶ Progesterone apparently has no effect on the lactogen content of the A.P. when administered in small or moderate dosages, but amounts well above the physiological level in rats (15.0 mg per day for 10 days) have increased it about 74% on a body weight basis.⁷ Selye

* Contribution from the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Journal Series No. 1040.

¹ Reece, R. P., and Turner, C. W., *Mo. Agr. Exp. Sta. Res. Bull.* No. 266, 1937.

² Lewis, A. A., and Turner, C. W., *Proc. Soc. Exp. Biol. and Med.*, 1941, 48, 439.

³ Meites, J., Trentin, J. J., and Turner, C. W., *Endocrinology*, 1942, 31, 607.

⁴ Meites, J., and Turner, C. W., *Endocrinology*, 1942, 30, 711.

⁵ Meites, J., and Turner, C. W., *Proc. Soc. Exp. Biol. and Med.*, 1942, 49, 190.

⁶ Reece, R. P., and Mixer, J. P., *Proc. Soc. Exp. Biol. and Med.*, 1939, 40, 66.

has shown that many steroid hormones can exhibit common "folliculoid" properties when administered in sufficient dosages,⁸ and this may well account for the action of progesterone on hypophyseal lactogen. From a cytological point of view it is interesting to note that estrogens and testosterone propionate have been shown to increase the number and secretory activity of the eosinophilic cells of the anterior pituitary,⁹⁻¹³ which are agreed to be the source of lactogenic hormone in both mammals¹⁴⁻¹⁶ and pigeons.¹⁷ The ability of sex hormones, particularly estrogens, to increase lactogen in the anterior pituitary is also reflected in the greater quantities of lactogenic hormone found in the blood of rabbits after estrone administration⁵ and in the initiation of milk secretion in a number of mammals following estrogen administration.^{4,18} The authors have advanced the theory that estrogen is the factor normally responsible for stimulating the secretion of pituitary lactogen and initiating milk secretion at the time of parturition.⁴

Crop secretion in pigeons is analagous to milk secretion in mammals and both processes furnish the means for supplying nutrition to the young during the critical period after birth. Would the sex hormones, therefore, stimulate pituitary lactogenic secretion in

pigeons as in mammals? Furthermore, if these steroids could increase the lactogen content of the pigeon pituitary, would it also be reflected in the initiation of crop gland secretion?

Experimental. Groups of 16 to 20 mature pigeons of both sexes were injected subcutaneously over the pectoral muscles daily for a 10-day period. These birds were kept together in groups of 8 to 10 in a cage. All the sex hormones were dissolved in corn oil and injected in 0.1 cc volumes. On the day after the last injection the birds were killed, weighed, sexed, and the pituitaries and crop glands were removed. The crop glands were washed clean, freed from adhering fat tissue, blotted dry between towel papers and weighed. They were then examined under light for evidence of visual proliferation. In the case of the diethylstilbestrol group and its controls, which constitute a preliminary experiment performed in 1942, the crop glands were not weighed but only examined for visual proliferation.

The pituitaries of each group were macerated with a small mortar and pestle, suspended in a definite volume of distilled water and injected intradermally over the crop glands of 10 common pigeons in 0.1 cc volumes for 4 days. The equivalent of 6 male or 4 female pigeon pituitaries was given to 10 birds, and each group of pigeon pituitaries was directly compared with another by injecting over both sides of the crop gland. The crop glands were rated for lactogenic response by the Reece-Turner method.¹

Results. It will be seen that in the preliminary experiment performed in 1942, 250 μ g of diethylstilbestrol did not increase the lactogen content of the pituitary or initiate crop milk secretion in 13 female pigeons. In the 1946 series, total dosages of 1,000 I.U. estrone, 10 mg progesterone or 5 mg testosterone propionate[†] caused no significant

¹ Reece, R. P., and Bivins, J. A., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 582.

⁸ Clarke, E., and Selye, H., *J. Pharm. and Exp. Therap.*, 1943, **78**, 187.

⁹ Foster, C. L., *J. Endocrinology*, 1942, **3**, 79.

¹⁰ Baker, B. L., and Everett, N. B., *Endocrinology*, 1944, **34**, 254.

¹¹ Zeckwer, I. T., *Science*, 1944, **100**, 123.

¹² Wolfe, J. M., *Endocrinology*, 1941, **29**, 969.

¹³ Wainman, P., Reese, J. D., and Koneff, A. A., *Endocrinology*, 1942, **31**, 303.

¹⁴ Azimov, G. I., and Altman, A. D., *Comptes Rendus de l'Acad. des Sciences de l'URSS*, 1938, **20**, 621.

¹⁵ Friedman, M. H., and Hall, S., *25th Annual Meeting and Scientific Session, Assn. for Study of Int. Secretions*, 1941, p. 10.

¹⁶ Smelser, G. K., *Endocrinology*, 1944, **34**, 39.

¹⁷ Schooley, J. P., and Riddle, O., *Am. J. Anat.*, 1938, **62**, 313.

¹⁸ Folley, S. J., and Malpress, F. H., *J. Endocrinology*, 1944, **4**, 1.

[†] We are indebted to Dr. D. F. Green of Merck & Co., Rahway, N.J., for the diethylstilbestrol; to Dr. D. W. MacCorquodale of The Abbott Research Labs., North Chicago, Ill., for the estrone; and to Dr. F. E. Houghton of Ciba Pharmaceutical Products, Inc., Summit, N.J., for the progesterone and testosterone propionate used in this study.

change in the lactogen content of the pituitary, and there was no evidence of visual proliferation or weight increase in the crop glands. The differences in the pituitary lactogen content between the 1942 and 1946 female control pigeons may be due to the fact that the former pigeon pituitaries were assayed in the early fall while the latter were assayed in mid-winter. As in a previous report on the lactogen content in the pituitaries of common pigeons,¹⁹ it was found that the female pituitary contains considerably more lactogen than the male. None of the sex hormones increased the pituitary weight of the pigeons.

Discussion. There appears to be an inverse relationship between the secretion of gonadal hormones and the formation of crop milk in pigeons. Thus, it has been demonstrated that (1) the testes and ovaries decrease in size during incubation and brooding, when crop secretion normally occurs,²⁰⁻²² and (2) the administration of lactogenic hormone markedly reduces the size of the pigeon gonads.^{23,24} With the exception of one report in which nonhormonal factors favorable to broodiness were introduced,²⁵ the injection of sex hormones has proven ineffective in stimulating crop secretion in pigeons.^{26,27} The mechanism responsible for the secretion and release of lactogen from the pigeon hypophysis may depend chiefly on psychic and

TABLE I. Effect of Sex Hormones on Pituitary Prolactin and Crop Glands of Common Pigeons.

Treatment	Sex	Total No. of pigeons	Avg body wt, g	Avg pituitary wt, mg	Avg crop wt, g	Avg crop wt		No. of visual crop responses	Avg No. lactogen units*	Avg No. lactogen units	
						100 g body wt	per pit.			100 g body wt	mg pit. tissue
Controls	♀	8	276	4.32			2.00	0		.72	.46
	♀	13	292	4.13			2.31	0		.79	.56
Controls	1♀	8	311	3.71	2.20	.70	1.62	0		.52	.43
	3M	10	326	3.44	2.47		.85	0		.26	.25
5 mg Testosterone propionate	2♀	11	285	3.82	2.22	.77	1.75	0		.69	.46
	4M	6	311	3.20	2.23	.71	1.12	0		.36	.35
10 mg Progesterone	2♀	9	284	3.32	2.11	.74	1.50	0		.53	.45
	3M	7	326	3.28	2.52	.77	.85	0		.26	.25
1000 I.U. Estrone	1♀	10	295	3.61	1.79	.60	2.06	0		.69	.54
	4M	7	336	3.45	2.22	.66	1.21	0		.36	.35

* Reece-Turner lactogen units = 1 International Unit (Meites, J., Bergmann, A. J., and Turner, C. W., *Endocrinology*, 1941, **28**, 707). 1-2-3-4. These groups were compared with each other for lactogenic hormone by injecting their pituitaries over both sides of the crop glands in the same 10 pigeons.

¹⁹ Hurst, V., Meites, J., and Turner, C. W., *Proc. Soc. Exp. Biol. and Med.*, 1943, **53**, 89.

²⁰ Champy, C., and Colle, P., *C. R. Soc. Biol.*, 1919, **82**, 227.

²¹ Riddle, O., Smith, G. C., and Benedict, F. G., *Am. J. Physiol.*, 1933, **105**, 482.

²² Schooley, J. P., and Riddle, O., *Anat. Rec. (Suppl.)*, 1936, **67**, 51.

²³ Riddle, O., and Bates, R. W., *Endocrinology*, 1933, **17**, 689.

²⁴ Bates, R. W., Lahr, E. L., and Riddle, O., *Am. J. Physiol.*, 1935, **111**, 361.

²⁵ Riddle, O., and Lahr, E. L., *Endocrinology*, 1944, **35**, 255.

²⁶ Patel, M. D., *Physiol. Zool.*, 1936, **19**, 129.

²⁷ Riddle, O., Bates, R. W., Miller, R. A., Lahr, E. L., Smith, G. C., Dunham, H. H., and Opdyke, D. F., *Carnegie Inst. Washington Year Book No.* 39, 1940, p. 221.

environmental rather than hormonal stimuli.²⁰ It seems clear from our study that the injection of sex hormones alone are ineffective in augmenting lactogen output by the pituitary or initiating crop milk secretion in pigeons.

It appears remarkable that the pigeon pituitary should be refractory to the administration of sex hormones, particularly estrogens which are such powerful activators of lactogenic hormone secretion in the pituitaries of mammals. Neither do estrogens increase the weight of the pigeon pituitary as in mammals. It would be of interest to determine what effect the sex hormones have on the

cytology of the pigeon hypophysis.

Summary. The effects of estrone, diethylstilbestrol, progesterone and testosterone-propionate were determined on the lactogen content and weight of the pituitary, and on the proliferation and weight of the crop glands of common pigeons of both sexes. The results were all negative. It is concluded that the pigeon pituitary, unlike the mammalian, is refractory to the administration of sex hormones and is not stimulated by them to increase the secretion of lactogenic hormone.

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Statistical Evaluation of Growth Curves.

CARROL S. WEIL. (Introduced by W. S. McEllroy.)

From the Multiple Fellowship on Chemical Hygiene, Mellon Institute, Pittsburgh, Penn.

A problem that frequently confronts research workers in the biological sciences is the comparison of growth curves of groups of experimental animals subjected to different treatments. The customary practice has been that of visual estimation of significant differences from a plot of weight against time (Fig. 1), or the application of the "t" test to the mean weights at a particular time. These conventional methods of evaluation

often give an ambiguous or unconvincing analysis of the results.

The purpose of this paper is to demonstrate how time-weight data (growth curves) have been treated more critically in a comparative study of exposure of rats to vapors

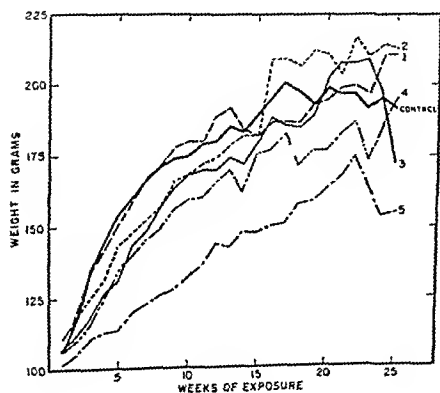


FIG. 1.

Combined growth curves of male and female rats.

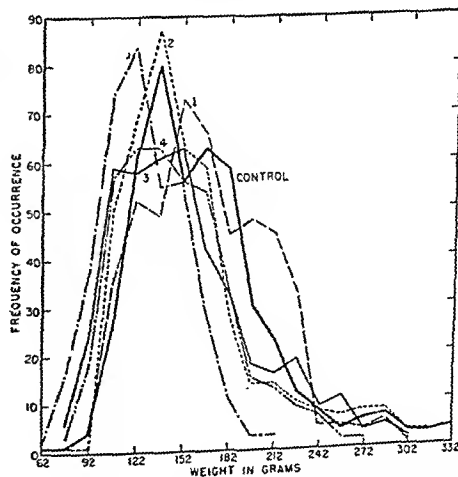


FIG. 2.

Curves showing the frequency of occurrence of individual weights for male and female rats, throughout the period of treatment.

TABLE I.
Summary of Data Showing Differences in Weight Response.

Group	No. of rat wts	Mean wt	Standard dev.	" t_0 " at 12 wks exposure	Probability level for 12 wk " t_0 "	$3\sigma/d$	$\chi^2 = \sum \left(\frac{(fo - ft)^2}{ft} \right)$	Chi square where	Probability level for chi square
Combined Weights of Male and Female Rats.									
1	464	168.4	40.6	0.72	0.50	3.74	19.8	0.14	—
Control	451	166.1	45.8	—	—	—	—	—	—
2	434	157.3	47.7	0.44	0.65	1.07	16.8	0.97	0.97
3	429	154.0	46.0	0.72	0.50	0.77*	31.1	0.008*	0.008*
4	395	147.7	36.9	1.03	0.30	0.46*	31.4	0.002*	0.002*
5	371	127.4	25.4	2.94	0.005*	0.20*	126.6	<0.001*	<0.001*
Weights of Male Rats.									
1	220	195.2	48.8	0.05	0.95	2.08	7.6	0.66	—
Control	209	188.0	54.5	—	—	—	—	—	—
3	227	169.2	54.8	0.54	0.60	0.84*	17.3	0.24	0.24
2	234	167.3	53.0	0.64	0.54	0.74*	9.7	0.70	0.70
4	180	157.1	44.2	1.03	0.32	0.49*	24.0	0.013*	0.013*
5	200	133.0	28.1	2.77	0.014*	0.23*	72.5	<0.001*	<0.001*
Weights of Female Rats.									
1	244	158.0	31.4	1.74	0.11	0.66†	21.4	0.003†	—
Control	242	146.6	23.6	—	—	—	—	—	—
4	215	140.0	27.1	0.12	0.91	1.09	9.8	0.20	0.20
3	202	137.1	24.0	0.35	0.73	0.72*	14.8	0.01*	0.01*
2	210	135.1	20.0	0.82	0.43	0.53*	17.6	0.004*	0.004*
5	171	119.4	25.6	2.18	0.04*	0.27*	57.6	<0.001*	<0.001*

* Decrease from control group is statistically significant.

† Increase above "

of a series of 5 analogous halogenated hydrocarbons. Each group consisted of about 30 animals, with males and females in approximately equal numbers; the sexes being kept separately. These groups were exposed on alternate days for 25 weeks, and each animal was weighed weekly. A control group was exposed to air alone. Fig. 1 shows the combined growth curves of the male and female rats as normally plotted, with time the abscissa and weight the ordinate. With the exception of Group 5, it is impossible to designate which group does or does not vary significantly from the control. The "t" test was applied to the weights of the 12th week of exposure as at this time the majority of the original animals were still alive. Again, with the exception of Group 5, nothing of significance was found. As a solution to the problem, the weight data were expressed in the form of frequency distributions and the weight curves were compared as one treats normal curves of other biological data. Each individual weekly weight of each rat alive at the time of weighing was considered a point in the distribution. Separate frequency distributions were prepared for the male and the female rats. These data were combined to show the overall effect of the materials. Fig. 2 illustrates the shape of these curves, where weight is the abscissa and the frequency of occurrence of each weight is the ordinate.

With the data in the form of frequency distributions, statistical comparison of the curves is simple and straight-forward. The method of chi square reveals similar probability levels of significance, as does the ratio of 3 times the standard error of the difference, to the difference between the mean weights. In each case exposed rats were compared to controls. A summary of the chi square and "t" data is presented in Ta-

ble I. The coefficient of skewness was calculated for the distributions of the control groups, and was found to be 0.48 for the combined male and female group, 0.19 for the male and 0.55 for the female controls. These values justify the use of normal statistics.

Discussion. The use of chi square is more valid for the comparison of weight-response curves than is a test of differences between means. In the chi square test the frequency with which a given weight range occurs in the treated group is directly compared to the frequency with which the same weight range occurs in the control group and there is made no arbitrary selection of a particular period for weight evaluation as is done in the "t" test. It was necessary to combine some weight classes at the extremities of distributions where the rats did not attain the spread in range of weights of the controls and where the theoretical frequencies of the classes were less than 5.

The use of a frequency distribution, in which each individual weekly weight is a point, yields a sufficient amount of valid data to make a good comparison of the weights of comparatively small groups of animals. In Table I the values for the groups of animals are listed in decreasing order of the mean weights. A remarkable relationship may be seen between the rank of the groups by this method of listing and by the magnitude of chi square and the $3\sigma d/d$ ratio.

Summary. By the application of frequency distributions to growth data, familiar statistical comparisons may be applied to determine whether significant differences exist. Previously no valid method was available to compare adequately growth curves in their entirety. The use of the chi square test on the distributions is deemed the method of preference for showing such differences.

Effect of Insulin Hypoglycemia and Glucose on Various Amino Acids in Blood of Mental Patients.

MEYER M. HARRIS AND RUTH S. HARRIS.

From the Department of Internal Medicine, New York State Psychiatric Institute and Hospital, New York City.

In previous publications^{1,2} we reported that the level in the blood of the total amino acids and also of glutamine is markedly depressed during the insulin hypoglycemic shock treatment of mental patients. It was also shown that the administration of glucose² produces a similar effect although it is not so marked as that produced during insulin hypoglycemic shock.

As part of an investigation regarding the metabolism of various amino acids in mental patients, determinations were made of the effect of insulin and of the administration of glucose on the level in the blood of the following amino acids: arginine, histidine, leucine, lysine, phenylalanine, tryptophane and valine.

It is known that the metabolism of some specific amino acids may be disturbed in certain clinical conditions as for example, phenylalanine in oligophrenia phenylpyruvica.^{3,4} Gjessing⁵ and others have reported on alterations in nitrogen metabolism in certain types of periodic psychotic episodes. In some pathological states certain amino acids may serve some special functions as for example, methionine in the protection of the liver against some poisons.^{6,7} Furthermore it is known that some amino acids can be synthe-

sized in the animal organism in amounts adequate for its needs while others are essential and must be supplied in the diet for normal nutrition.⁸

No study regarding a group of individual amino acids has thus far been carried out in mental patients owing to the lack of adequate methods. The recent development of microbiological methods has made such study feasible.

Procedure and Methods. A group of 15 patients undergoing or about to undergo a course of insulin hypoglycemic shock treatment was used for this study. The patients were about 16 hours postabsorptive when the observations were begun. They were kept in bed during the entire period of study.

After a preliminary specimen of venous blood was taken, the patient was given either a dose of insulin subcutaneously which produced hypoglycemic coma, or 75 g of glucose in 200 ml of tap water which was flavored with lemon juice. Nothing was administered to 4 patients who were used as controls.

Blood was withdrawn from the anterior cubital vein in an oiled syringe and discharged into a flask containing heparin. The blood samples were taken to the laboratory immediately and centrifuged for 10 minutes and the plasma separated. The plasma was again centrifuged for 5 minutes to remove any stray red blood cells. The plasma proteins were then immediately precipitated with tungstic acid according to the method of Hier and Bergeim.⁹ The precipitate was removed by centrifugation and filtration. The filtrate was adjusted to pH 7 with 1N NaOH using the Beckman pH meter.

¹ Harris, M. M., Blalock, J. R., and Horwitz, W. A., *Arch. Neurol. and Psychiat.*, 1938, **40**, 116.

² Harris, M. M., Roth, R. T., and Harris, R. S., *J. Clin. Invest.*, 1943, **22**, 569.

³ Penrose, L. S., and Quastel, J. H., *Bioch. J.*, 1937, **31**, 266.

⁴ Jervis, G. A., Block, R. J., Bolling, D., and Kaniz, E., *J. Biol. Chem.*, 1940, **134**, 105.

⁵ Gjessing, R., *Arch. f. Psychiat. u. Nervenkr.*, 1935, **104**, 355.

⁶ Miller, L. L., Ross, J. F., and Whipple, G. H., *Am. J. Med. Sc.*, 1940, **200**, 739.

⁷ Miller, L. L., and Whipple, G. H., *J. Exp. Med.*, 1942, **76**, 421.

⁸ Sahyun, M., *Outline of the Amino Acids and Proteins*, 1944, p. 223.

⁹ Hier, S. W., and Bergeim, O., *J. Biol. Chem.*, 1945, **161**, 717.

Each determination was carried out in triplicate. *Leuconostoc mesenteroides* P-60 and the improved medium D of Dunn *et al.*¹⁰ were used for the determination of lysine and histidine. Tryptophane was determined according to Dunn *et al.*¹¹ using *Lactobacillus arabinosis*. The medium of Hac, Snell and Williams¹² and *Lactobacillus casei* were used for the determination of arginine. Leucine and valine were determined with the medium of Shankman¹³ and *Lactobacillus arabinosis*. Phenylalanine was determined with the medium of Dunn *et al.*¹⁴ and *Lactobacillus casei*.

One milliliter of basal medium was employed in all the determinations except that of histidine. For the determination of lysine, valine and phenylalanine, 1 ml of blood filtrate was used and for arginine, tryptophane, and leucine only 0.5 ml of filtrate plus 0.5 ml of water was employed. For the determination of histidine it was found necessary to increase the volume of basal medium to 2.5 ml to which was added 1 ml of filtrate and 1.5 ml of water.

A set of standards was run with each determination instead of depending upon standard curves. This acted as a check as to any changes in the behavior of the microorganisms.

Tryptophane and histidine were determined turbidometrically with the Coleman spectrophotometer with the light band set at 540. Electrometric titration, with the Beckman pH meter, of the acid production was used for the determination of the other amino acids.

The total amino acid nitrogen was determined by the colorimetric method of Frame *et al.*^{15,16}

The blood sugar was determined by the colorimetric method of Somogyi.¹⁷

The Coleman spectrophotometer was used for all colorimetric determinations.

Observations. Table I shows the effect of insulin hypoglycemia on the level of the total amino acid nitrogen and of various amino acids in the blood plasma. It will be noted that the total amino acids and all of the individual amino acids studied thus far, were significantly depressed during insulin hypoglycemic coma. However, the various amino acids were not depressed to the same degree. This is indicated by the marked variations in the percentage drop from the initial levels of the various amino acids in the same patient (Table I). Thus for example, in patient (J.B., No. 1) lysine is depressed 70% whereas the level of the total amino acids is depressed only 33%. It will also be seen that the levels of leucine and lysine are the 2 amino acids most markedly depressed during insulin hypoglycemic coma. The drop in the levels of leucine ranged from 63 to 81% of the initial level, and that of lysine ranged from 47 to 77%. With a few exceptions, the percentage drop in the level of the individual amino acids was equal or greater than the drop in the level of the total amino acids (Table I).

The initial level of the amino acids appeared to bear no relationship to the dose of insulin required to produce hypoglycemic coma, and the extent of the drop of the level of the amino acids was not directly related to the size of the dose of insulin which was administered.

Table II shows the effect of the administration of 75 g of glucose, orally, on the blood sugar level and the corresponding changes in the level of the total amino acids and of some of the individual amino acids in the blood plasma. The greatest drop in the level of the amino acids occurred in the first hour after the administration of glucose, at which time the level of the blood sugar was the highest. There was, with an occasional exception, a further drop at the end of 2 hours at which time the blood sugar level was either

¹⁰ Dunn, M. S., Shankman, S., Camien, M. N., Frankl, W., and Rockland, L. B., *J. Biol. Chem.*, 1944, **156**, 703.

¹¹ Dunn, M. S., Schott, H. F., Frankl, W., and Rockland, L. B., *J. Biol. Chem.*, 1945, **157**, 387.

¹² Hac, L. R., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, 1945, **159**, 273.

¹³ Shankman, S., *J. Biol. Chem.*, 1943, **150**, 305.

¹⁴ Dunn, M. S., Shankman, S., and Camien, M. N., *J. Biol. Chem.*, 1945, **161**, 643.

¹⁵ Frame, E. G., Russell, J. A., and Wilhelm, A. E., *J. Biol. Chem.*, 1943, **149**, 255.

¹⁶ Russell, J. A., *J. Biol. Chem.*, 1944, **156**, 467.

¹⁷ Somogyi, M., *J. Biol. Chem.*, 1945, **160**, 69.

TABLE I.
Effect of Insulin Hypoglycemia on the Level of Various Amino Acids in the Blood Plasma.

Patient and No.	Sex	Age, yr	Wt., kg	Dose of insulin units	Total amino acid nitrogen mg/100 ml		Arginine γ /ml		Histidine γ /ml		Leucine γ /ml		Lysine γ /ml		Tryptophane γ /ml		Valine γ /ml	
					*B	*A	B	A	B	A	B	A	B	A	B	A	B	A
J.B. 1	M	29	81	220	6.4	4.3 (33.4)	23.8 (43)	13.5 (43)	15.3 (55)	6.9 (70)	57.6 (64)	20.5 (64)	25.4 (70)	7.6 (70)	14.6 (38)	9.0 (38)	34. (49)	17.4 (49)
J.M. 2	M	29	61	160	7.2	4.1 (43)	22.1 (39)	13.5 (39)	16.5 (59)	6.7 (77)	63.4 (75)	15.8 (75)	32.8 (77)	7.6 (77)	15.8 (39)	9.6 (39)	35.6 (62)	13.5 (62)
W.B. 3	M	34	66	150	6.4	4.0 (38)	21.4 (40)	12.8 (40)	12.3 (43)	7.0 (47)	52.8 (63)	19.6 (63)	29.1 (47)	15.3 (59)	11.3 (19)	9.2 (19)	45.9 (39)	27.9 (39)
J.B. 4	M	32	91	290	7.3	4.8 (34)	31. (52)	16.2 (52)	16.4 (51)	8.0 (59)	60.6 (72)	17.0 (72)	42. (59)	17.4 (59)	13.7 (34)	9.1 (34)	46.5 (40)	27.8 (40)
A.J. 5	M	29	72	340	6.0	3.9 (35)	23.8 (50)	11.8 (50)	13.0 (42)	7.5 (81)	52. (79)	10.8 (79)	15.7 (67)	5.2 (67)	12.8 (34)	8.4 (34)	41.2 (44)	23.1 (44)
A.M. 6	M	23	65	130	7.2	3.8 (37)	29.6 (64)	10.8 (64)	14.3 (43)	8.1 (81)	57. (81)	11.1 (81)	28.9 (74)	7.4 (74)	12.8 (47)	6.8 (47)	44.4 (48)	23.1 (48)
R.A. 7	F	21	72	100	8.9	4.1 (54)	25.5 (65)	8.7 (65)	13.4 (60)	6.7 (86)	62.8 (88)	20.1 (88)	36.6 (86)	11.9 (86)	21.6 (35)	14. (35)	38.2 (53)	17.8 (53)
L.S. 8	F	31	49	120	7.8	4.8 (39)	33.9 (51)	16.8 (51)	12.1 (37)	7.6 (79)	54.9 (79)	14.7 (79)	35.0 (61)	13.5 (61)	16.5 (39)	10. (39)	34.5 (58)	14.6 (58)
P.D.G. 12	M	19	91	0	6.5	6.6	30.9	28.2	12.2	13.1	63.6	57.0	26.5	26.5	14.1	13.0	44.3	40.4
R.L. 13	M	18	83	0	6.5	6.4	24.3	24.9	10.0	11.3	61.8	59.4	21.2	24.0	12.4	12.2	39.6	38.1
N.D. 14	F	18	46	0	5.9	5.8	22.0	22.2	14.4	13.7	51.9	54.9	34.5	33.5	15.1	12.7	35.7	34.5
R.J. 15	M	23	51	0	5.9	5.9	24.9	24.6	13.1	13.2	49.8	48.9	21.7	23.6	16.8	10.0	31.8	27.3

* B = Level before the administration of insulin.

* A = Level 3½ hours after insulin, at which time patients were in hypoglycemic coma. Controls, without insulin, show the effect of 3½ hours rest in bed.

† = Figures in parentheses indicate the depression of the level of the amino acids in per cent of the initial level.

‡ = Phenylalanine was determined in this patient and was 7.2 γ and 7.1 γ per ml before and after 3½ hours of rest, respectively.

TABLE II.
Effect of the Oral Administration of Glucose on the Level of Various Amino Acids in the Blood Plasma.

Patient and case No.	Sex	Age yr	Wt kg	Time hr	Blood sugar mg/100 ml	Total amino acid mg/100 ml	Arginine γ /ml	Histidine γ /ml	Leucine γ /ml	Lysine γ /ml	Phenylalanine γ /ml	Tryptophane γ /ml	Valine γ /ml
S.W. 9	M	15	72	0*	92	6.1	24.4	14.2	55.8	24.8	—	14.0	33.6
				1	152	5.3 (13)†	20.4 (16)	12.2 (14)	42.0 (25)	21.3 (14)	—	11.5 (18)	26.7 (21)
				2	80	5.1 (16)	18.0 (26)	11.3 (20)	32.1 (43)	19.8 (20)	—	11.3 (19)	21.8 (35)
J.F. 10	M	20	67	3½	79	5.1 (16)	19.2 (21)	12.0 (15)	37.5 (33)	19.1 (23)	—	10.7 (24)	21.1 (37)
				0	90	6.4	29.4	12.5	63.8	30.3	6.5	12.2	45.2
				1	127	5.3 (17)	19.5 (34)	11.0 (12)	48.4 (24)	24.5 (19)	5.6 (14)	10.2 (16)	33.6 (26)
				2	109	5.4 (16)	17.7 (40)	10.4 (17)	37.8 (41)	24.5 (19)	5.1 (22)	9.2 (24)	26.7 (41)
C.C. 11	F	17	52	3½	75	5.3 (17)	17.2 (41)	11.0 (12)	38.1 (40)	25.2 (17)	4.5 (31)	10.1 (16)	25.1 (44)
				0	85	5.4	22.8	12.7	37.8	19.4	5.1	9.4	24.0
				1	138	4.8 (11)	15.6 (32)	12.2 (4)	25.2 (33)	16.1 (17)	4.5 (12)	8.5 (10)	17.5 (27)
				2	—	—	—	—	—	—	—	—	—
				3½	74	4.4 (19)	13.8 (39)	12.0 (6)	25.6 (32)	17.4 (10)	5.1 (0)	9.9 (-5)	15.0 (38)

* Time interval after the administration of glucose.

† Figures in parentheses represent the depression of the amino acids in per cent of the initial level.

lower or higher than the initial postabsorptive level. After 3½ hours the blood sugar fell below the initial level while the amino acids all remained significantly depressed below the initial level and tended to show either a slight rise or fall from the level reached at the 2-hour period.

The findings in 4 control patients who did not receive insulin are shown at the bottom of Table I. After 3½ hours the levels of the amino acids in the blood of these patients either remained practically unaltered or tended to rise or fall by a few per cent from the initial postabsorptive level. The findings were in marked contrast to those observed after the administration of insulin or glucose. The controls also might be considered an added check on the methods used in this study.

Discussion. Our report in a previous publication² that there was a marked depression in the level of glutamine and total amino acids in the blood plasma of patients receiving insulin hypoglycemic shock treatment, was confirmed by Hamilton¹⁸ in experiments on dogs. Since he found that the percentage drop of glutamine and of the total amino acids was the same he interpreted these findings as indicating that the drop in the level of glutamine was due merely to a diffusion of the amino acids from the blood into the tissues. We have suggested¹⁹ that other interpretations of the data were possible and have discussed the possibilities in a previous publication.²

The findings in this study indicate that various amino acids in the blood differ in the extent to which they are affected during insulin hypoglycemic coma. Such differences are also found following the oral administration of glucose. It is apparent that some pronounced alterations in nitrogen metabolism, other than the uniform diffusion of amino acids from the blood into tissues, must occur which would account for the results obtained.

¹⁸ Hamilton, P. B., *J. Biol. Chem.*, 1945, **158**, 397.

¹⁹ Harris, M. M., Roth, R. T., and Harris, R. S., *J. Nerv. and Ment. Dis.*, 1945, **102**, 466.

Bouckaert and his coworkers²⁰ and Mirsky²¹ were of the opinion, from their studies on nephrectomized, hepatectomized, and eviscerated animals, that insulin inhibits deamination by the liver and also affects the synthesis and hydrolysis of proteins in the various tissues of the body. Since the injection of insulin into eviscerated animals resulted in the disappearance of endogenous amino acids from the blood stream, Mirsky²¹ favored the view that this was due to an increase in synthesis rather than a decrease in hydrolysis of proteins in the peripheral tissues. Frame and Russell²² have recently carried out similar studies on eviscerated rats and confirmed the findings. They point out that it is not known at present whether insulin promotes synthesis of tissue proteins from blood amino acids or inhibits its breakdown.

From the standpoint of the possible mechanisms involved it is of interest to analyze the changes observed in the level of the various amino acids in the blood as a result of insulin and glucose administration. It will be observed that, with the exception of arginine, and histidine, the amino acids thus far studied fall into the group of so-called essential amino acids. These amino acids may be synthesized in the body to only a very limited extent or not at all.⁸ This should simplify, somewhat, the analysis of the changes observed. The figure for total amino acid nitrogen represents both essential and nonessential amino acids in the blood plasma.

It will be seen from Table I that in the controls the level of the amino acids shows little change during the 3½ hours of observation in the postabsorptive state. Following the administration of insulin or glucose (Tables I and II) there is a significant drop, but to a variable degree, in the levels of the various amino acids.

As the controls indicate, the patients, during the 3½ hours of observation, are approximately in a state of dynamic equilibrium as

far as the level of the various amino acids in the blood are concerned. That is, the amino acids supplied to the blood as a result of protein hydrolysis or amino acid synthesis is about balanced by those removed from the blood for deamination and oxidation or protein or polypeptide synthesis.

Since insulin or glucose tends to spare protein metabolism one should expect a decrease in deamination and oxidation of amino acids. This is in keeping with the findings of Mirsky²¹ and Bouckaert, and his co-workers²⁰ in their animal experiments. However, a decrease in deamination and oxidation of amino acids should tend to raise the level of amino acids in the blood. Since an appreciable drop in the level of amino acids is observed following glucose or insulin, an inhibition of hydrolysis of tissue proteins or an increase in the synthesis of polypeptides or proteins probably occurs to account for the findings.

If, in the various patients, insulin or glucose inhibited the hydrolysis of the same type of storage protein or increased the synthesis of some polypeptide or protein of similar composition, then the ratios of the amounts of the various amino acids disappearing from the blood stream should be similar in the different patients. This tendency occurs for some of the amino acids in some of the patients. However, there are many significant deviations. Thus, for example, patient (J.B., No. 1) in Table I shows a drop of 37 γ of leucine and 18 γ of lysine per ml of plasma. This is a ratio of leucine to lysine of approximately 2. In patient (A.J., No. 5), leucine dropped 41 γ and lysine only 10.5 γ which gives a ratio of leucine to lysine of approximately 4. Thus the mechanisms which depress the level of the amino acids, thrown into action as a result of the insulin hypoglycemia, may not be identical in the 2 patients. This marked difference in the handling of essential amino acids may be highly significant.

In the case of the nonessential amino acids, arginine and histidine, it will be seen that there are marked differences in the ratios of the amounts of these amino acids which disappear from the blood plasma. Thus, for example, patient (J.M., No. 2) showed a drop

²⁰ Bouckaert, J. B., and de Duve, Chr., *Physiol. Rev.*, 1947, **27**, 39.

²¹ Mirsky, I. A., *Am. J. Physiol.*, 1938, **124**, 569.

²² Frame, E. G., and Russell, J. A., *Endocr.*, 1946, **39**, 420.

of 8.6 γ of arginine and 9.8 γ of histidine per ml of plasma while in patient (A.M., No. 6) 18.8 γ of arginine and 6.2 γ of histidine disappeared from the plasma. Thus, while more than twice the amount of arginine disappeared in the latter as compared with the former patient, as regards histidine the situation was reversed. Thus the extent of the relative effect of insulin hypoglycemia on arginine and histidine formation and utilization must be significantly different in these patients. The studies of previous investigators regarding the effect on the level of total amino acids in the blood failed to reveal the complexity of the metabolic processes which require consideration. General speculation as to hydrolysis and synthesis of tissue protein to account for the marked effects of insulin and glucose administration would appear to be a rather crude approach to complex metabolic processes.

In some patients the oral administration of glucose produced as marked a depression in the level of valine and arginine as that seen in insulin hypoglycemic coma. (See patient J.F., No. 10, Table II). However, the effect on the other amino acids was not comparable. This points to some special effects of insulin aside from the supply and utilization of glucose on the metabolism of some of the amino acids. This seems to be especially marked as regards leucine and lysine of the amino acids thus far investigated. In this connection it may be of interest to point out that Steele and his co-workers²³ recently have found that leucine is one of a few amino

acids which is not excreted in human urine, either free or combined, except under very special conditions. This occurs in spite of the comparatively high level of leucine in the blood. These findings may point to some special or unique function of leucine in the nitrogen metabolism of man. This will be investigated further.

The possible relation of the amino acids to clinical conditions will be the subject of another paper.

Summary. 1. The effect of insulin hypoglycemic coma and of the oral administration of glucose on the level of various amino acids in the blood have been investigated in 15 mental patients.

2. The level in the blood of all the amino acids, thus far investigated, decreases to a variable extent during insulin hypoglycemia and also after glucose administration.

3. Insulin hypoglycemia produced a more marked effect on the level of blood amino acids than did the administration of glucose. Valine appeared to be an exception in some patients.

4. Leucine and lysine showed the most marked changes of the amino acids thus far investigated.

5. The total amino acids does not reflect the extent of the changes in the blood of the level of the individual amino acids.

6. The possible mechanisms which may account for the changes are discussed.

²³ Steele, B. F., Sauberlich, H. E., Reynolds, M. S., and Baumann, C. A., *J. Nutrition*, 1947, 33, 209.

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Studies on Cigarette Smoke Irritation. I. Determination of Edema-Producing Properties of Certain Types of Cigarette Tobaccos.

P. S. LARSON, H. B. HAAC, AND J. K. FINNEGAN.

From the Department of Pharmacology, Medical College of Virginia, Richmond.

We described¹ a method for the quantita-

tive evaluation of cigarette smoke irritation, as judged by its edema-producing properties, based on gravimetric determination of the increase in moisture content of the upper

¹ Finnegan, J. K., Fordham, Doris, Larson, P. S., and Haug, H. B., *J. Pharm. and Exp. Therap.*, 1947, 80, 115.

TABLE I.
Comparative Edema-Producing Properties of Certain Types of Cigarette Tobaccos.

Type of cigarette	No. of observations	Mean of ratio differences*	Mean of % increase in moisture
A. Smyrna (Turkish)	20	0.56	11.82
B. Bright	20	0.96	21.76
C. Maryland	20	1.38	30.76
D. Samsoun (Turkish)	20	1.47	33.79
E. Burley	20	1.71	37.12
F. Blend of A-E	50	1.47	31.14

* Ratios for exposed tissues minus ratios for control tissues.

TABLE II.
Significance of the Differences Observed Between the Edema-Producing Properties of the Types of Cigarette Tobaccos Listed in Table I.

Comparison	P value*	Comparison	P value	Comparison	P value
A vs B	.41	B vs C	.31	C vs E	.39
A vs C	.01	B vs D	.29	C vs F	.76
A vs D	.02	B vs E	.10	D vs E	.70
A vs E	.002	B vs F	.12	D vs F	.98
A vs F	.002	C vs D	.82	E vs F	.44

* Probability values of .05 and less are usually held to indicate statistical significance; .01 and less indicate a high degree of significance.

palpebral conjunctiva of the rabbit eye following its exposure to cigarette smoke. The method is briefly as follows:

Using a smoking machine of the type described by Bradford, Harlan and Hanmer² which draws a 35 ml puff once per minute, 3 puffs are directed through a suitable eye cup placed over the widely opened eye of a morphinized rabbit. When one eye only of each rabbit is exposed, the other serving as a control, we have named the technic "Procedure A." Where both eyes of each rabbit are exposed, thereby permitting comparison of 2 cigarettes on the same animal, the technic is called "Procedure B." In both procedures, following exposure to the smoke, a one-hour period is allowed for edema formation. The rabbit is then sacrificed, the upper lid membrane of each eye excised, weighed, dried in an oven to constant weight, and reweighed. From the wet and dry weights the ratio of moisture to dry weight is calculated and, in the case of Procedure A, the per cent increase in water in the exposed membrane over that in the control membrane is determined. In a series of such determinations

involving 2 or more groups of cigarettes, the differences in the ratios between groups are evaluated statistically for significance by analysis of variance.

In the present study, we have applied Procedure A to an investigation of a blend of cigarette-tobacco types in an effort to evaluate the role of each in the production of cigarette smoke irritants of the edema-producing type.

Experimental. Six groups of cigarettes were prepared, one containing only Smyrna (Turkish) tobacco, one containing only Samsoun (Turkish) tobacco, one containing only Bright tobaccos, one containing only Maryland tobaccos, one containing only Burley tobaccos, and one containing a blend of all of these tobaccos. All were brought to comparable moisture content (11.5-12.5%) by placing them in atmospheres of suitable humidity. From each group, cigarettes were selected for smoking purposes on the basis of comparable resistance to air flow through them when subjected to a slight constant negative pressure at one end. Fifty of the blended cigarettes and 20 from each of the other groups were tested by Procedure A and the results are recorded in Table I and the significance of differences in Table II.

² Bradford, J., Harlan, W., and Hanmer, H. R., *Ind. Eng. Chem.*, 1936, **28**, 836.

Discussion. From Tables I and II it is apparent that the smoke from certain types of cigarette tobaccos tends to be lower in edema-producing irritants than that from other types, and that in a blend of these tobaccos these irritants appear to be additive.

In our original description of the method here used¹ and throughout the presentation of the present study, we have stressed the fact that the only irritants measured by this technic are those that produce edema. That this is not necessarily related to the sensations of subjective irritation experienced by a smoker was strikingly brought out in the present study in the case of the cigarettes made from Burley tobaccos alone. As measured by edema-producing irritants alone this cigarette was not significantly more irritating than the blended cigarette, yet the sensations experienced by the smoker were quite the opposite. Whereas, the average smoker had no difficulty in making a deep inhalation of the smoke from the blended cigarette, a similar inhalation of smoke from the Burley cigarette left him unable to draw a second breath for

an uncomfortably long period. It is apparent from this that substances which produce a degree of subjective irritation disproportionately greater than that which might be expected on the basis of their edema-producing properties, may be present in cigarette smoke. Development of a method capable of quantitative measurement of the subjective sensation of irritation to complement the measurement of edema-producing irritants so that a more complete picture may be obtained is greatly to be desired.

Conclusions. 1. Smoke from different types of cigarette tobaccos may differ significantly in edema-producing irritants. 2. Constituents are present in cigarette smoke, from at least certain types of tobaccos, which produce a degree of subjective irritation disproportionately greater than that which might be expected on the basis of their edema-producing properties.

We are indebted to Mrs. Sarah Guerry for technical assistance in performing the reported experiments.

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New Formula for Dilution Curve of Plasma Prothrombin. Normal Standards and Changes in Pathologic Conditions.

S. RAPOPORT.

From the Children's Hospital Research Foundation, and the Department of Pediatrics, College of Medicine, University of Cincinnati, Cincinnati.

The various one-stage methods for the measurement of prothrombin time used at present, differ little from the procedure originally employed.¹ They are based on the assumption that the clotting time of plasma is dependent solely on its prothrombin content, if an excess of thromboplastin and optimal amounts of calcium are provided; constancy of the rate of conversion of prothrombin to thrombin is assumed. As source of

thromboplastin an extract of rabbit brain, or the venom of the Russel viper, are most commonly used.

In all procedures the quantitative comparison of abnormal with normal plasma meets with as yet unresolved difficulties, which have occasioned a variety of methods of assay. The simplest assumption,² that the clotting

¹ Quick, A. J., Stanley-Brown, M., and Bancroft, F. W., *Am. J. M. Sc.*, 1935, **190**, 501; Quick, A. J., *J. A. M. A.*, 1938, **110**, 1658.

² Ziffren, S. E., Owen, C. A., Hoffman, G. R., and Smith, H. P., *Am. J. Clin. Path., Tech. Supp.*, 1940, **4**, 13; Ziffren, S. E., Owen, C. A., Hoffman, G. R., and Smith, H. P., *Proc. Soc. Exp. Biol. and Med.*, 1939, **40**, 595; Govan, C. D., *J. Pediat.*, 1946, **29**, 629.

time of plasma is linearly proportional to its prothrombin content, is obviously incorrect,³ since it can be shown that the clotting time of normal plasma on dilution follows a curve resembling a hyperbola in shape.^{1,4} This curve shares with the hyperbola the property that the product of the 2 coordinates, clotting time in seconds and percentage of plasma concentration, tends to be constant but differs from a hyperbola in 2 respects: (1) a plasma percentage over 100 *a priori* cannot exist, and (2) the approach to infinity of clotting time in the region of low plasma concentrations is not truly asymptotic; beyond a certain dilution, plasma will no longer clot at all. In comparing abnormal plasma with the normal, generally the assumption has been made, either expressly or implicitly, that the clotting properties of abnormal plasma can be expressed in terms of a dilution of normal plasma. Often the prothrombin content has been expressed as per cent of normal, based on a curve established for serial dilutions of normal plasma. If the assumption underlying such a procedure were correct, dilution curves of normal and abnormal plasmas could be made to coincide by proper linear shifting of their curves along one of the coordinates. For instance, an abnormal plasma which undiluted might have a clotting time corresponding to a 50% concentration of normal plasma, on dilution with an equal volume of saline should give a clotting time corresponding to a 25% concentration of normal plasma. Serious doubts concerning the validity of this premise have been expressed, and it has been demonstrated that the dilution curves of pathic and normal plasmas usually cannot be superimposed, indicating

that not only their starting points, but also their slopes differ.^{5,6} One group of investigators, for purposes of assay of prothrombin-lowering substances, plotted the logarithm of the clotting time versus the logarithm of plasma concentration.⁵ This method also is based on the assumption that the curves of normal and abnormal plasma have the same slope. No proof was adduced for this premise.

Some observers have preferred either a 12.5 or a 25% concentration of plasma for the routine measurement of prothrombin,^{6,7} since in states of prothrombin deficiency the clotting time of plasma increases more on dilution than normally. One investigator,⁸ noting considerable variation in the difference between the clotting times of undiluted plasma and a 1:8 plasma dilution in various clinical conditions, proposed that this difference was a more reliable index of the prothrombin activity than the clotting time of either undiluted or diluted plasma alone; when the difference between undiluted and diluted plasma was increased a state of hypoprothrombinemia was indicated, and either hyperprothrombinemia, or an increase of coagulation-inhibitors, when the difference was reduced.

Finally, a formula describing the dilution curve of normal plasma of rabbit and man has been given as follows:⁹

$$t = a + k/c$$

where t is the clotting time of any given concentration of plasma, c is the concentration of plasma, and a and k are constants. a is the intercept at infinite concentration, while at 100% concentration $t - a = k/100$. a was found to be 5.1 for rabbit and 10.3 for human plasma, while the k values were 139 and 354, respectively. This formula, indicating direct proportionality between the clot-

³ Quick, A. J., *The Hemorrhagic Diseases and the Physiology of Hemostasis*, Springfield, Ill., 1942, C. C. Thomas.

⁴ Page, R. C., de Beer, E. J., and Orr, M. L., *J. Lab. and Clin. Med.*, 1941, **27**, 197; Quick, A. J., *Am. J. Physiol.*, 1941, **132**, 239.

⁵ Campbell, H. A., Smith, W. K., Roberts, W. L., and Link, K. P., *J. Biol. Chem.*, 1941, **138**, 1.

⁶ Overman, R. S., Stahlmann, M. A., Sullivan, W. R., Huebner, C. F., Campbell, H. A., and Link, K. P., *J. Biol. Chem.*, 1942, **142**, 941.

⁷ Brambel, C. E., and Loker, F. F., *Proc. Soc. Exp. Biol. and Med.*, 1943, **53**, 218.

⁸ Shapiro, S., *Exp. Med. and Surg.*, 1944, **2**, 103; Shapiro, S., Sherwin, B., Redish, N., and Campbell, H. A., *Proc. Soc. Exp. Biol. and Med.*, 1942, **50**, 85.

⁹ Quick, A. J., and Leu, M., *J. Biol. Chem.*, 1937, **119**, LXXXI; Quick, A. J., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 788.

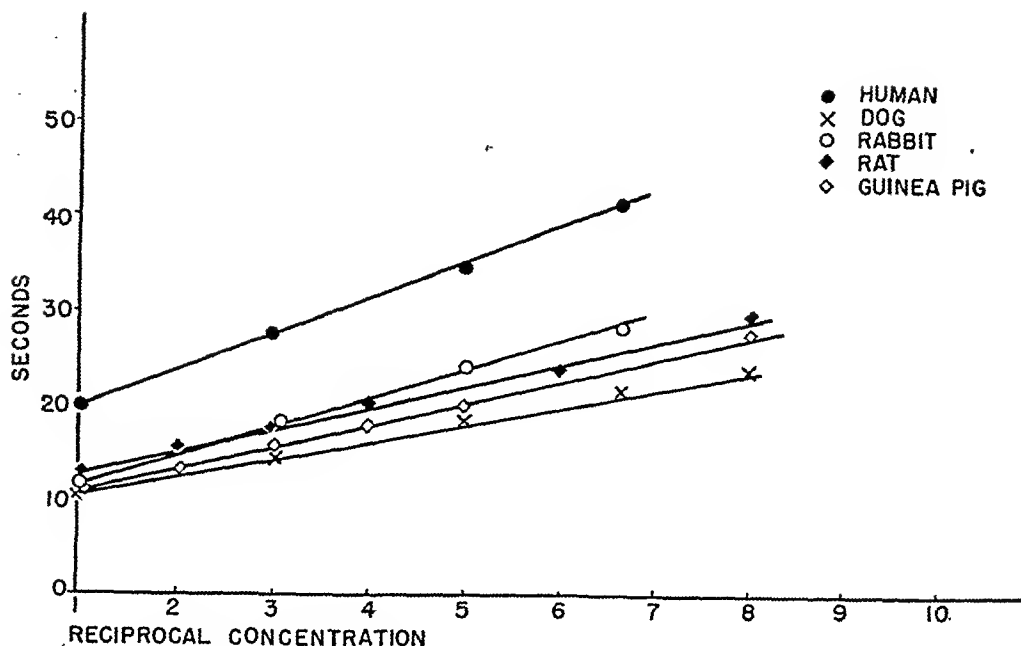


Fig. 1.

Prothrombin time and dilution constant of the blood plasma of man, dog, rabbit, rat and guinea pig.

ting time and $1/c$, the reciprocal of the concentration, fits the experimental finding that for any given plasma the product of clotting time and dilution tends to be constant. The authors did not use the formula for the description of abnormal plasma.

In the following it will be shown that the prothrombin time of normal as well as abnormal plasma of man, dog, rabbit, rat and guinea pig may be expressed by the following formula:

$$t = t_1 + k/(\text{dilution}^* - 1)$$

where t is the clotting time in seconds of any given plasma dilution,* t_1 that of undiluted plasma, and k is a proportionality factor, the dilution constant, which indicates the increase of clotting time per unit increment of reciprocal concentration. This formula, the equation of a straight line, indicates as do some formulas previously mentioned, direct proportionality between the reciprocal of plasma dilution and the clotting time but differs from them in the choice of the constants.

* Dilution = the ratio of the final volume of the plasma-saline mixture to the original volume of plasma.

Both t_1 and k are constants for any given plasma, but vary among different samples and under pathologic conditions. Considered analytically the 2 constants, representing the intercept and the slope of the line, are sufficient to describe the values of serial dilutions of any given plasma. In this communication are presented the normal standard values of t_1 and k for human, dog and rabbit plasma, 2 examples of the behavior of the constants in experimentally-produced hypoprothrombinemia, and a survey of their variations in clinical conditions.

Experimental. Freshly drawn blood, collected by venipuncture with precautions to avoid hemolysis and foaming, was delivered into test tubes measuring 10 x 75 mm and graduated at 1 cc, which contained 0.1 cc of 0.1 molar sodium oxalate. After gentle but thorough mixing the tubes were centrifuged for 5 minutes at 2000 r.p.m. The supernatant plasma was drawn off and the prothrombin time was determined according to the slightly modified method of Fullerton¹⁰ as follows: an 0.15 cc aliquot of the oxalated

¹⁰ Fullerton, H. W., *Lancet*, 1940, 2, 195.

TABLE I.
Mean Normal Values for the Prothrombin Time of Undiluted Plasma (t_1) and the Dilution Constant of Man, Dog, and Rabbit.

All values are given in seconds.							
Species	No. of samples	Undiluted plasma			Dilution constant		
		Mean	S.D.*	S.E.*	Mean	S.D.*	S.E.*
Man	37	19.9	1.2	0.2	3.8	0.9	0.2
Dog	22	11.4	1.4	0.3	1.8	0.5	0.2
Rabbit	10	11.4	1.4	0.4	3.2	0.5	0.4

* S.D. = standard deviation; S.E. = standard error.

plasma was measured into a small test tube and placed for one minute in an all-glass water bath permitting close observation at 37°. Then an equal amount of a similarly prewarmed mixture of thromboplastin and calcium chloride was added, and with constant shaking of the tube in the water bath the time for the formation of a solid fibrin clot recorded. Clotting times were also measured on various dilutions of plasma prepared with 0.9% sodium chloride solution. All determinations were performed in duplicate. The mixture of thromboplastin with calcium chloride was prepared by dissolving 0.5 mg of Russel-viper venom (Stypven, Burroughs-Wellcome Laboratories, 9 East 41st Street, New York, N. Y.) in 10 cc of 0.025 molar calcium chloride solution. The mixture was found stable for at least one month when kept in the refrigerator between tests. No significant differences between different batches of venom were ever noted over a period of 4 years, except in 1945-46 when owing to war-time shortages difficulties were experienced by the manufacturer in the preparation of a satisfactory product.

Results. 1. Normal values. In Fig. 1 are presented representative data on individual samples of normal human, dog, rabbit, rat and guinea pig plasma. As can be seen, a straight line relationship obtains for the range of dilutions studied. Fig. 1 also illustrates the fact that there is little relation between the clotting time of undiluted plasma and the dilution constant among different species. For instance it is apparent that the clotting times of the undiluted plasma of dog and rabbit are similar while the slopes of the dilution curves differ widely. On the other hand, rabbit and human plasma give

similar dilution constants, despite the difference in the clotting time of the undiluted plasmas.

In Table I are given the mean values of the clotting time of undiluted plasma and the dilution constant, for human, dog and rabbit plasma together with their statistical description. For human plasma, if one accepts the conventional limits of ± 2 standard deviations as the limits of normal variation, a range of 17.5-22.2 seconds for the undiluted plasma and 2.0-5.6 for the dilution constant may be given. A proportional range is found for the plasmas of dog and rabbit.

The formula appears valid over a dilution range up to approximately 1:10 for normal

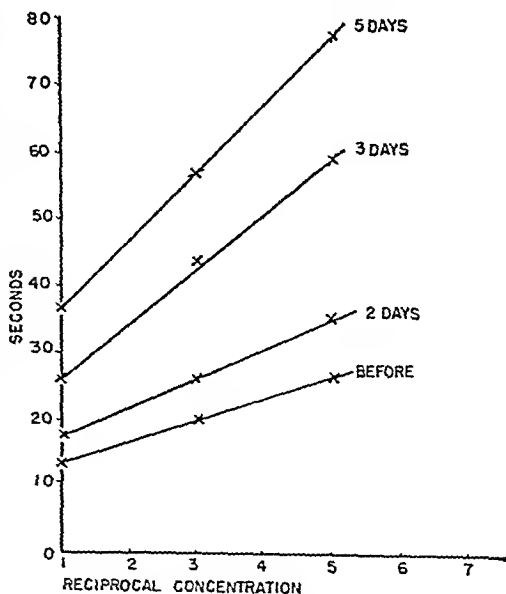


Fig. 2.
The prothrombin time of a rabbit following the subcutaneous injection of 0.5 cc per kg of methyl salicylate.

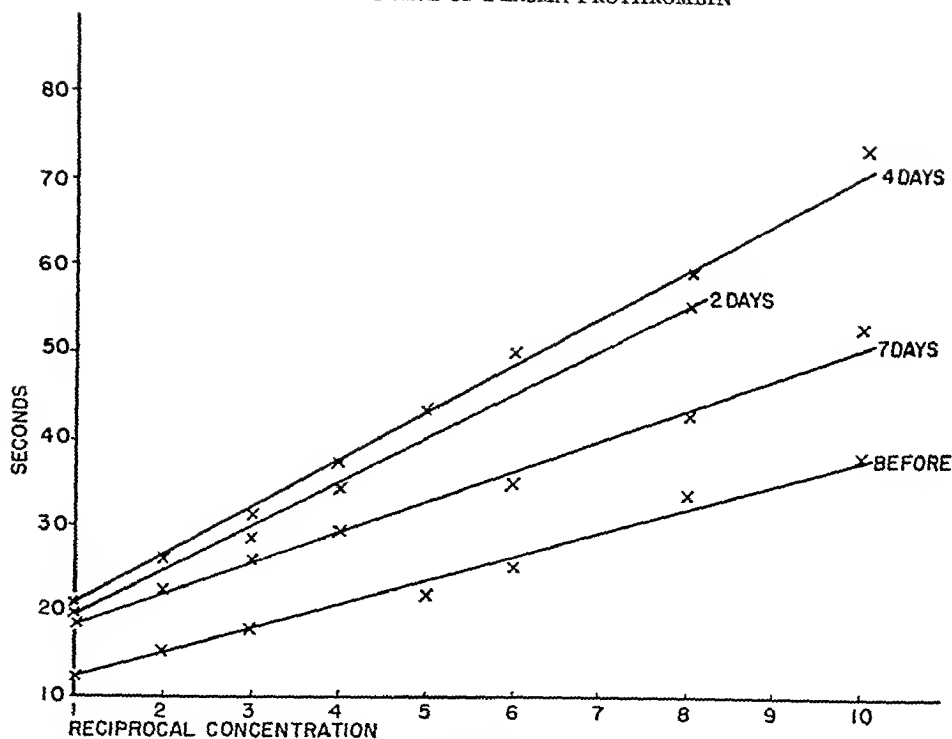


Fig. 3.

Changes of the prothrombin time of a rabbit following the intravenous injection of 2 mg per kg of dicumarol.

human and approximately 1:20 for dog plasma. On further dilution disproportionate lengthening of the clotting time occurs. In plasma with prolonged clotting time the proportionality range is reduced, suggesting the existence of an upper limit of approximately 70-80 seconds for the validity of the formula.

2. Changes in experimentally-produced hypoprothrombinemia. Two examples of hypoprothrombinemia produced in rabbits by the administration of dicumarol and methylsalicylate are presented in Figs. 2 and 3. It may be seen that in both experiments the undiluted plasma clotting time as well as the dilution constant increased. Such data indicate that both indices are related to the prothrombin content of the plasma, and that the curves of abnormal and normal plasma cannot be superimposed.

3. Changes in clinical conditions. The purpose of this section is not so much to present a compilation of changes of prothrombin time in disease, as to indicate the relation of the clotting time of undiluted plasma to the dilu-

tion constant. The patients for the most part were children. The data are divided into 2 groups on the basis of the dilution constant. In Table II are presented in order of increasing value cases of elevation of the dilution constant. They are recruited mostly from 3 groups: (1) cases of deficiency of vitamin K, (2) liver damage of various kinds, and (3) bleeding for extended periods of time. A fourth group of cases, patients on salicylate medication, is separately presented in Table IV.

It may be seen that there are interesting parallels as well as discrepancies in the behavior of the clotting time of undiluted plasma (t_1) and the dilution constant. In all cases of vitamin K deficiency, with marked prolongation of t_1 the dilution constant also was elevated. On the other hand in patients with severe hepatic damage the dilution constant was increased independently of the changes of undiluted plasma, an observation which has proved to be of diagnostic and prognostic value. The discrepancy between

TABLE II.
Cases of Prolonged Dilution Constant. (Listed in order of increasing value).

Case No.	Plasma prothrombin time Undiluted plasma (t_1) seconds	Dilution constant seconds	Diagnosis
1.	45.5	6.0	Aplastic anemia, bleeding profusely
2.	30.9	6.1	Chronic diarrhea
3.	25.7	7.2	Syphilitic hepatitis
4.	18.4	7.3	Congenital atresia of the bile ducts
5.	36.3	7.3	Lymphosarcoma, bleeding
6.	29.5	7.3	Histoplasmosis
7.	36.1	7.5	Infectious hepatitis, convalescing
8.	28.5	7.6	" " "
9.	33.9	7.6	Chronic diarrhea
10.	35.8	7.7	Intrahepatic biliary obstruction
11.	21.0	8.2	Cirrhosis of liver
12.	26.6	8.6	Rheumatic fever, cardiac decomposition with hepatic congestion
13.	26.7	8.7	Thrombocytopenic purpura
14.	15.0	8.8	Erythroblastosis fetalis
15.	27.2	9.0	Banti's disease
16.	18.4	9.3	Hemophilia, prolonged bleeding from gastro- enteric tract
17.	28.1	9.9	Rheumatic fever with congestion of liver
18.	28.7	9.9	Erythroblastosis fetalis, severe
19.	24.6	10.0	Typhoid fever, severe, 1 day pre-mortem
20.	30.9	10.0	Hemophilia, epistaxis of 5 days' duration
21.	43.0	10.1	Erythroblastosis fetalis, severe anemia
22.	31.2	10.4	Banti's disease
23.	30.1	10.5	Congenital atresia of bile ducts, bleeding
24.	19.6	10.8	Cirrhosis of liver
25.	15.1	10.9	" " "
26.	34.0	10.9	Chronic diarrhea
27.	25.1	11.2	Infectious hepatitis of long duration
28.	23.2	12.4	Diarrhea
29.	21.5	13.8	Hepato-renal syndrome of lupus erythematosus
30.	32.3	14.3	Letterer-Siwe's disease, 1 day pre-mortem
31.	51.2	14.4	Acute infectious hepatitis with subacute liver atrophy
	27.9	15.5	1 day after vitamin K, 1 day pre-mortem
32.	20.0	14.9	Cirrhosis of liver
33.	29.4	16.4	Erythroblastosis fetalis, severe, 8 hours pre-mortem
34.	21.6	19.1	Erythroblastosis fetalis
35.	52.6	≈32	Chronic diarrhea
36.	61.8	≈37	" "
37.	66.8	≈44	Hemorrhagic disease of the newborn
38.	80.2	≈56	Chronic diarrhea
39.	76.8	≈75	" "

the 2 indices in cases of hepatic damage as compared with their parallelism in vitamin K deficiency, suggests that humoral clotting factors other than prothrombin may play a role in hepatic disorders. The data on cases of prolonged bleeding, here presented, indicate that the immediately available supply of prothrombin, or vitamin K, may be exhausted. Thus patients with hemophilia may also develop hypoprothrombinemia (see cases 16 and 20). The data on the effect of salicylate medication in the usual dosage (0.1-0.15 g per kg body weight) on patients

with rheumatic fever suggest that with parallel changes of the undiluted plasma and the dilution constant, the latter is a more sensitive indicator of the changes of prothrombin.

The cases of decrease of the dilution constant are drawn from a more heterogeneous material than those of prolongation. In Table III 3 groups appear prominent: (1) cases of infectious hepatitis in an early stage, (2) cases of the nephrotic syndrome, and (3) cases of rheumatic fever. Also represented are cases of purpura, leukemia, and Rocky Mountain Spotted Fever, disorders with bleeding

TABLE III.
Cases of Reduction of the Dilution Constant. (Listed in order of increasing value.)

Case No.	Plasma prothrombin time Undiluted plasma (t_1) seconds	Dilution constant seconds	Diagnosis
40.	17.7	—0.4	Post-infectious state, with high serum agglutinin titer
41.	21.0	0.0	Non-thrombocytopenic purpura
42.	22.5	0.4	Nephrotic syndrome
43.	30.0	0.4	„ „
44.	16.0	0.5	Infectious hepatitis
45.	23.8	0.5	Symptomatic purpura
46.	19.9	0.6	Nephrotic syndrome
47.	17.1	0.7	Post-tonsillectomy bleeding
48.	21.7	0.8	Infectious hepatitis
49.	30.6	0.8	Acute lymphatic leukemia
50.	16.4	1.3	Infectious hepatitis
51.	20.7	1.4	Nephrotic syndrome
52.	15.2	1.5	Rheumatic fever
53.	29.0	1.5	Rheumatoid arthritis
54.	18.3	1.7	Nephrotic syndrome
55.	22.5	1.7	Rocky Mountain spotted fever
56.	14.8	1.8	Acute lymphatic leukemia
57.	16.6	1.8	Rocky Mountain spotted fever
58.	21.7	1.8	Infectious hepatitis
59.	14.7	2.0	Sickle cell anemia in hemolytic crisis
60.	19.9	2.0	Rocky Mountain spotted fever
61.	20.8	2.0	Nephrotic syndrome
62.	14.4	2.1	Rheumatic fever
63.	16.1	2.1	„ „
64.	16.4	2.1	Infectious hepatitis
65.	22.3	2.1	Rheumatic fever

from the small vessels as a common feature. Another group, cases of patients receiving vitamin K, is presented in Table V.

Again, a widely varying relationship between t_1 and the dilution constant is evident. While both indices changed in a parallel manner in some patients with infectious hepatitis and in those receiving vitamin K, marked discrepancies were found among the other patients. Of particular interest is the combination of a considerable prolongation of t_1 with a marked reduction of the dilution constant (see cases 43, 49 and 53). Whether the reduced dilution constant reflects an actual increase of prothrombin content or is the result of an increase in the amount of auxiliary clotting factors cannot be said at the present. Reduction of both t_1 and the dilution constant suggests strongly the existence of a state of true hyperprothrombinemia but additional effects of activators and inhibitors of clotting have to be invoked in cases where the 2 indices differ from each other.

Discussion. It may be well to point out

that no definite independent biological meaning has been assigned to the 2 constants in the expression for prothrombin time. The data on hypoprothrombinemia, experimental as well as clinical, suggest that the constants, not truly independent of each other, change in a parallel manner. On the other hand, the observations on different species and on the clinical material illustrate marked and well defined discrepancies between the 2 indices. These differences may be related to variations in the amount of auxiliary factors of clotting rather than of prothrombin.

Clinically of greatest interest is the apparent usefulness of the dilution constant as an indicator of hepatic damage. In infectious hepatitis decreases as well as increases of the dilution constant occur, suggesting the occurrence of a period of stimulation, followed by suppression, of hepatic function. Similar changes in cases of bleeding may be due to stimulation followed by exhaustion of prothrombin formation. Most of the conditions in which a decrease of the dilution constant was observed are characterized by altera-

TABLE IV.
Effect of Salicylate on Dilution Curve of Plasma Prothrombin.

Case No.	Plasma prothrombin time Undiluted plasma (t_1) seconds	Dilution constant seconds	Comments
66.	20.1	4.9	Before
	26.6	9.1	2 days salicylate
	25.5	9.7	4 " "
	26.8	10.6	6 " "
	25.8	11.5	8 " "
	23.2	9.6	2 " stopped
67.	20.2	5.6	Before
	24.7	6.4	2 days salicylate
	22.9	6.8	4 " "
	22.1	5.7	2 " stopped
68.	16.2	2.2	Before
	24.1	5.0	4 days salicylate
	27.5	6.8	7 " "
	19.6	4.7	5 " after
69.	21.0	3.7	Before
	23.9	5.8	3 days salicylate
	23.9	6.7	6 " "
	20.2	4.7	4 " after
70.	20.8	5.2	Before
	31.1	10.3	4 days salicylate
71.	22.0	4.7	Before
	22.9	7.1	4 days salicylate
	20.5	5.5	2 " after
72.	19.1	4.7	Before
	21.3	6.0	2 days salicylate
	20.3	6.9	6 " "
73.	19.7	3.5	Before
	21.4	6.4	2 days salicylate
	22.6	7.2	7 " "
74.	20.2	2.4	Before
	27.6	9.6	12 days salicylate
	22.0	2.0	10 " after

TABLE V.
The Effect of Vitamin K Medication on Dilution Constant.

Caso No.	Plasma prothrombin time Undiluted plasma (t_1) seconds	Dilution constant seconds	Comments
10.	35.8	7.7	Intrahepatic biliary obstruction
	16.1	0.9	2 days after Vit. K
37.	66.8	≈ 4.4	Hemorrhagic disease of new born
	16.2	1.4	1 day after Vit. K
39.	76.8	≈ 7.5	Chronic diarrhea
	14.7	1.7	2 days after Vit. K
27.	25.1	11.2	Infectious hepatitis of long duration
	19.2	2.0	2 days after Vit. K
75.	15.4	1.4	Erythroblastosis fetalis 1 day after Vit. K
76.	16.3	1.8	Chronic diarrhea 1 day after Vit. K

tions in the distribution of plasma protein and increases of fibrinogen. Such patients may experience a general humoral stimulation in which clotting factors participate.

Summary. A new formula expressing the

dilution curve of plasma prothrombin is presented. Normal standards and examples of change in experimental clinical conditions are given.

15837

Inhibitory Effect of Dibenamine on Vasoconstrictor Substances.

H. HAIMOVICI.*

From the Laboratories of The Mount Sinai Hospital, New York City.

Recently Nickerson and Goodman¹ reported the results of the study of a new adrenolytic and sympathicolytic drug, dibenzyl- β -chloroethyl amine hydrochloride (dibenamine). They found that after the administration of this agent to dogs and cats, intravenous epinephrine in high doses causes a reversal of blood pressure (a fall of 20 to 40 mm Hg). In addition to the blocking and reversing of the excitatory adrenergic response, dibenamine had the ability to prevent epinephrine-induced cardiac irregularities in dogs sensitized by cyclopropane.² Most of the studies on dibenamine reported above deal with responses of heart rate, blood pressure, nictitating membrane, pupillary reactions.

Because of the possible clinical usefulness of dibenamine in angiospastic conditions associated with peripheral vascular diseases, causalgic states or hypertension,[†] an investigation of vasomotor responses to this agent, as expressed in terms of peripheral blood flow, seemed of interest. The present report deals with a study of the inhibitory effect of dibenamine on vasoconstrictor substances.

Methods. All the experiments reported in this investigation were carried out in the

Laewen-Trendelenburg (L-T) preparation in frogs (*Rana pipiens*). This preparation is a sensitive indicator in testing vascular responses, and offers a simple tool for the analysis of vasomotor agents and their antagonists. The perfusate used was a solution containing 0.65% NaCl and 0.02% KCl. Two Mariotte bottles were connected by a Y-piece with the perfusion system, *i.e.* the rubber tubing leading to the aortic cannula. One bottle contained the plain perfusion solution, the other, added to the latter, dibenamine.[‡] This substance was used at various concentrations. Epinephrine hydrochloride, nicotine base (Eastman Kodak) and hypertensin (angiotonin)[§] were used in testing the vasoconstrictor-blocking ability of dibenamine. The injections of these vasopressor substances were made through the rubber tubing into the glass cannula inserted into the abdominal aorta of the frog. Their vasomotor activity was evaluated in terms of the output variations of drops per minute.

Results. The injection of a small amount of epinephrine (1/10-1 γ), nicotine (1-10 γ) or hypertensin (0.05-0.5 cc) in the L-T preparation induces a marked vasoconstriction as

* Elsa and William Menke Fellow.

This investigation was aided by a grant from the Peripheral Vascular Disease Research Fund.

¹ Nickerson, M., and Goodman, L. S., *Fed. Proc.*, 1946, 5, 194.

² Nickerson, M., Smith, S. M., and Goodman, L. S., *Fed. Proc.*, 1946, 5, 195.

[†] Clinical investigation of the action of dibenamine in such conditions is now in progress.

[‡] Dibenamine was supplied by Givaudan-Delawanna, Inc., Delawanna, N.J., through the courtesy of Dr. W. Gump.

[§] Hypertensin (angiotonin) was prepared from the interaction of Renin (Smaco) and sheep serum according to the method described by Sapirstein *et al.*³

³ Sapirstein, L. A., Reed, R. K., and Southard, F. D., Jr., *J. Lab. and Clin. Med.*, 1944, 29, 633.

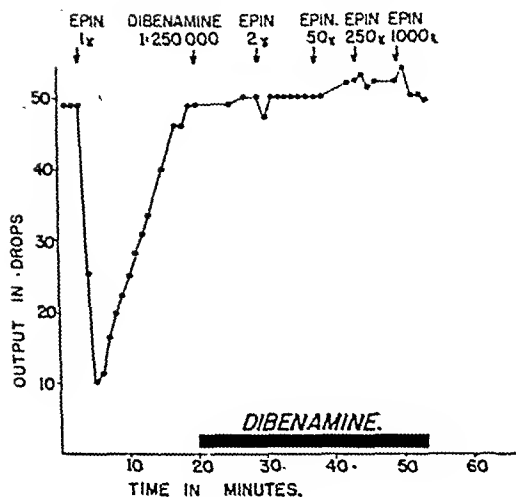


Fig. 1.

Inhibitory effect of dibenamine on the vasoconstrictor action of epinephrine. (The bar indicates the time of perfusion with dibenamine).

expressed by the reduction of the output (50-80%) and the duration of this effect ranging from 10 to 30 minutes or more, *i.e.* before the output returns to its initial level. Each individual preparation was first tested for its sensitivity to epinephrine as well as to the other pressor substances used in this investigation.

I. Effect of dibenamine on epinephrine.

(a) Dibenamine hydrochloride in a concentration of 1:2,500,000 or less inhibits the action of epinephrine only partially. In higher concentrations ranging from 1:1,000,000 to 1:250,000 or more, dibenamine inhibits the vasoconstrictor action of epinephrine completely within 5 to 10 minutes after the onset of the perfusion. The outstanding feature of this effect, as the perfusion with dibenamine progresses (20 to 30 minutes), is a complete inhibition of the response to even large amounts of epinephrine (50-1000 γ) (Fig. 1).

(b) Dibenamine hydrochloride was kept in solution together with epinephrine (100 γ of epinephrine in 50 cc of a solution of 1:250,000 dibenamine) *in vitro* for 30 to 60 minutes. When such epinephrine was tested after that lapse of time, its vasoconstrictor activity was essentially unaltered.

(c) According to the chemical constituents present in the perfusate, dibenamine ex-

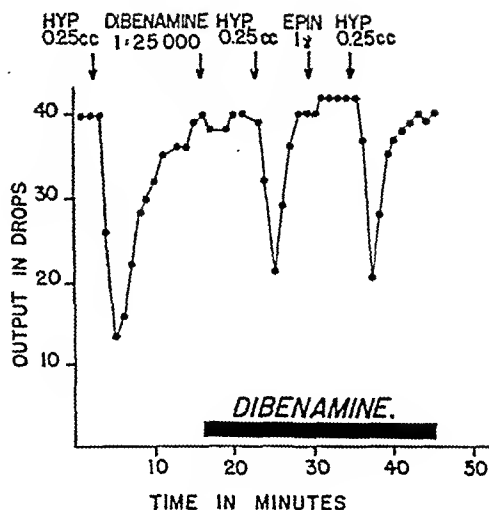


Fig. 2.

Effect of dibenamine on hypertensin. (The bar indicates the time of perfusion with dibenamine).

hibited a more or less complete inhibitory action on large amounts of epinephrine. The perfusing solution containing 0.65% NaCl and 0.02% KCl is the most suitable vehicle, if dibenamine is to show a complete blockade of large amounts (50-1000 γ) of epinephrine. The bicarbonate-free Tyrode solution is less suitable, for dibenamine does not then inhibit the vasoconstrictor action of large amounts of epinephrine completely. This seems to be due chiefly to the presence of calcium chloride. Indeed, when the perfusate contained 0.035% CaCl_2 in addition to 0.65% NaCl and 0.02% KCl, the adrenolytic action of dibenamine was greatly diminished for the large amounts of epinephrine.

II. *Effect of dibenamine on hypertensin (angiotonin).* Amounts of hypertensin ranging from 0.05 cc to 0.5 cc induce a marked and sustained constriction of the vascular bed of the frog's hind legs. 10 to 20 minutes after the onset of the perfusion with dibenamine, in concentrations ranging from 1:250,000 to 1:25,000, the vasoconstrictor action of hypertensin remains unchanged. After 40 to 50 minutes of perfusion its action is altered only slightly (Fig. 2).

III. *Effect of dibenamine on nicotine.* The injection of small amounts of nicotine (1-10 γ) in the L-T preparation elicits 2

major reactions: (a) twitchings of the thigh and leg muscles, (b) marked vasoconstriction.⁴ Within 30 to 45 minutes after the onset of the perfusion with dibenamine (1:250,000 to 1:25,000) when the action of large amounts (50-1000 γ) of epinephrine is completely blocked, nicotine still induces notable vasoconstriction and visible twitchings of the muscles.

Discussion. : Dibenamine hydrochloride used in concentrations of 1:1,000,000 to 1:25,000 prevents epinephrine-induced vasoconstriction as tested in the Laewen-Trendelenburg preparation. The protective effect afforded by this drug appears to be very potent, for it can block the action of very large amounts of epinephrine (1000 γ). This is practically a total adrenolytic effect. A comparative study with benzyl-imidazoline hydrochloride (priscol),^{||} another synthetic autonomic blocking agent,⁵ used under the same experimental conditions, exhibits a similar protective action but only for smaller amounts of epinephrine. This is in agreement with the results obtained by other investigators experimenting in intact animals.^{2,6} The inhibitory effect of dibenamine on epi-

nephrine does not seem to be due to a direct chemical interaction between the 2 molecules. The *in vitro* experiments reported above seem to support this view. In addition to being adrenolytic, dibenamine exhibits also a marked sympathicolytic action as shown by studies in the intact animal.^{1,2} Since dibenamine is an adrenolytic and sympathicolytic agent, its site of action is probably at the neuroeffector cells or between the postganglionic nerve endings and the latter.

Within 30 to 50 minutes after the onset of the perfusion with dibenamine, when the action of large amounts of epinephrine is completely inhibited, small amounts of both hypertensin and nicotine still exhibit a marked vasoconstrictor activity. These facts seem to indicate that dibenamine is either specifically adrenolytic or that these various vasoconstrictor substances do not have the same site of action.

Summary. 1. Dibenzyl- β -chloroethyl amine hydrochloride (dibenamine) inhibits the vasoconstrictor action of epinephrine, as tested in the Laewen-Trendelenburg preparation in the frog. Its adrenolytic action is very potent, for it blocks even large amounts of epinephrine (50-1000 γ). 2. When the action of large amounts of epinephrine is completely inhibited by dibenamine, both hypertensin and nicotine still exhibit a marked vasoconstrictor activity. 3. The peripheral locus of action of dibenamine seems to be at the neuroeffector cells or between the postganglionic nerve endings and the latter.

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Effect of Thiouracil and Estrogen on Lactogenic Hormone and Weight of Pituitaries of Rats.*

JOSEPH MEITES AND C. W. TURNER.

From the Department of Dairy Husbandry, University of Missouri, Columbia, Mo.

The weight of the hypophysis increases

subsequent to thyroidectomy,¹⁻⁴ and this may

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¹ Hammett, F. S., *Am. J. Anat.*, 1923, **32**, 37.

² Hammett, F. S., *Endocrinology*, 1926, **10**, 145.

³ Smelser, G. K., *Anat. Rec.*, 1939, **74**, 7.

be due to an augmentation of the basophil cells^{4,5} which are presumed to secrete thyrotropin.⁶⁻⁸ The acidophils, on the other hand, become markedly decreased following thyroidectomy.⁹ The administration of goitrogenic substances, like thyroidectomy, can produce similar effects on the hypophysis. Thus, they have been reported to increase the weight of the pituitary,¹⁰ increase and hypertrophy the basophil cells, and reduce the number and size of the eosinophils.¹¹⁻¹³

The acidophils of the hypophysis are the source of the lactogenic hormone,^{6,7,14} and hence it would be expected that this hormone would be reduced following thyroidectomy. However, the only 2 reports available on this subject are not in agreement. McQueen-Williams reported that thyroparathyroidectomy in male rats reduced the lactogen content of the pituitary,¹⁵ whereas Reineke, Bergman and Turner found no apparent change in the pituitary content of this hormone following thyroidectomy in young goats.¹⁶

It was thought that since thiouracil is similar in its effects to thyroidectomy, it would offer a considerable advantage in a restudy

of the relation of thyroid deficiency to the secretion of hypophyseal lactogenic hormone. In the rat it would make unnecessary the surgical removal of the thyroids, with the inevitable concomitant removal of the parathyroids which creates an additional deficiency in the animal. It was also considered of interest to determine the effects of estrogen in combination with thiouracil on the lactogen content of the pituitary. Estrogen has been shown to be a powerful stimulator of lactogenic secretion, greatly increasing its content in the pituitary,¹⁷⁻¹⁹ in the blood²⁰ and initiating milk secretion in several species.^{18,21,22} Would thiouracil inhibit the dynamic effect of estrogen on pituitary lactogenic secretion?

Experimental. The first experiment ran for 24 days, and was performed on young female albino rats of a standard strain we have been using for years.[†] The control rats (Group 1) received no special treatment while Group 2 was fed a ration containing 0.1% thiouracil[‡] during the 24-day period. Group 3 was given 100 I.U. estrone[‡] daily during the last 10 days of the 24-day period, while Group 4 received 0.1% thiouracil for the first 14 days, and thiouracil plus 100 I.U. estrone daily during the last 10 days of the experiment.

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[†] We are indebted to Dr. A. G. Hogan, Chairman of the Department of Agricultural Chemistry, University of Missouri, for a generous supply of these rats.

[‡] We wish to thank Dr. Mark Welsh of the Lederle Lab., Pearl River, N.Y., for the thiouracil; Dr. D. W. MacCorquodale of the Abbott Research Labs., North Chicago, Ill., for the estrone; and Dr. D. F. Green of Merck and Co., Rahway, N.J., for the diethylstilbestrol used in this study.

TABLE I.

Experiment 1. Normal Female Rats.									
Group	No.	Sex	Treatment	Avg body wt		Avg No. lactogen units*			Avg thyroid wt, 100 g B.W., mg
				1st day	24th day	per pit.	per 100 g body wt	per mg pit. tissue	
1	10	F	Controls	108	130	8.5	6.0	1.25	12.5
2	9	F	10.1% Thiouracil	109	129	5.5	4.2	0.61	57.6
3	10	F	2100 I.U. estrone daily	110	128	12.0	9.3	1.49	44.5 ± 2.85
4	10	F	20.1% Thiouracil + 100 I.U. estrone, 14th through 24th days	116	126	5.5	4.3	0.60	36.5 ± 3.51
Experiment 2. Castrate Male Rats.									
Group	No.	Sex	Treatment	Avg body wt		Avg pit. wt, mg	Avg pit. wt / 100 g B.W.	Avg thyroid wt, 100 g B.W., mg	Avg thyroid wt / 100 g B.W., mg
				1st day	21st day				
1	7	M	Controls	132	180	9.17	5.09†	10.6	6.5 ± .03
2	8	M	210 γ Diethylstilbestrol daily	129	130	7.81	6.02	12.8	9.5 ± .95
3	6	M	210 γ Diethylstilbestrol + 0.1% Thiouracil daily	128	134	11.60	8.69	53.1	38.8 ± 3.18

1-23 Groups assayed and compared with each other in same 10 pigeons.

* Reece-Turner units.¹⁷

† Standard error of the mean.

‡ Individual weights were not available for determining S.E.M.

The second experiment[§] ran for 21 days and included 3 groups of young male rats which had been castrated about 10 days earlier. Group 1 served as controls and received subcutaneous injections of .05 cc of corn oil daily. Group 2 was given injections of 10 μ g of diethylstilbestrol[‡] in corn oil daily while Group 3 similarly received 10 μ g of diethylstilbestrol by injection plus 0.1% thiouracil in the feed daily.

All the rats were killed by etherization, and the pituitaries and thyroids were removed and weighed to the nearest .01 mg. Each group of pituitaries was macerated with a small mortar and pestle, suspended in an exact volume of distilled water, and the equivalent of 2 female or 6 male pituitaries was injected intradermally over one side of the crop glands of 10 pigeons. Two different groups of pituitaries were assayed and compared with each other in the same 10 pigeons by injecting over each side of the crop gland. The crop glands were rated for lactogen units by the Reece-Turner method.¹⁷

Results. *Experiment 1.* The lactogenic hormone in the pituitaries of the thiouracil-treated rats (Group 2) was reduced below that in the controls (Group 1), averaging 4.2 units compared to 6.0 units per 100 g body weight. The rats which received estrone alone (Group 3) showed a considerable increase in pituitary lactogen over the controls, averaging 9.3 units per 100 g body weight. Group 4, which received the same amount of estrone but got thiouracil in addition, not only showed no increase in lactogenic hormone over the control rats but had as little (4.3 units per 100 g body weight) as in the animals treated with thiouracil alone.

The pituitaries of the thiouracil, and thiouracil plus estrone-treated groups were definitely heavier than in the control rats, both increasing about the same amount per 100 g of body weight. The thyroids of both these groups weighed much more than in the controls, but the increase in thyroid weight

was less in the group which received thiouracil plus estrone.

Experiment 2. It will be seen that in this experiment the lactogen content of the pituitaries of the thiouracil plus stilbestrol-treated rats (Group 3) rose just as much above the controls (Group 1) as the animals which received stilbestrol alone (Group 2). It must be pointed out that the Group 3 rats in this experiment do not correspond to the thiouracil plus estrone-treated group in the first experiment. The former received thiouracil and estrogen together during the entire 21-day period, while the latter got only thiouracil for the first 14 days and thiouracil with estrogen during the last 10 days of the 24-day period.

The pituitaries of the rats which were given thiouracil plus stilbestrol (Group 3) weighed considerably more than the animals treated with stilbestrol alone (Group 2) or the controls (Group 1). The average thyroid weight of the rats in Group 3 was increased approximately 5-fold over the controls.

Discussion. The reduction in the lactogen content of the pituitary following thiouracil treatment confirms the results reported by McQueen-Williams in thyroparathyroidectomized rats. This is believed to support the contention that the eosinophils, which are decreased following thyroidectomy or goitrogen treatment, are the source of lactogenic hormone. It is interesting that whereas the combination of thiouracil and estrogen in the first experiment did not evoke an increase in pituitary lactogen, it did do so in the second experiment. It is believed this difference can be accounted for in the somewhat different procedure followed with the 2 groups. Thus, the former received the estrogen only after a preliminary treatment with thiouracil for 2 weeks whereas the latter group received thiouracil and estrogen together during the entire length of the experiment. It would seem that in the first experiment the administration of thiouracil alone during a 2-week preliminary period reduced the eosinophils in the hypophysis to such a low level that they were rendered incapable of responding

[§] This was part of another experiment by V. Hurst, J. J. Trentin and the authors to determine the effects of thiouracil plus estrogen on mammary growth.

to subsequent estrogen stimulation and hence evoked no lactogen increase. In the second experiment on the other hand, the simultaneous administration of estrogen with thiouracil checked the depressing effect of the latter on the eosinophils and enabled the estrogen to evoke its normal increase of pituitary lactogen.

The increase in the pituitary weight following thiouracil treatment corroborates a similar report on thiourea-treated rats,¹⁰ and is in line with the findings in thyroidectomized animals. The recognized ability of estrogen to increase pituitary size was evident both in the estrone and diethylstilbestrol-injected rats. However, the greatest augmentation of pituitary weight occurred in the rats which received the above estrogens in combination with thiouracil.

Summary. The administration of 0.1% thiouracil in the feed for 24 days to young

female rats reduced the lactogenic hormone content of the pituitary below that in normal rats. The proven ability of estrogen to increase the lactogen content of the pituitary remained unimpaired in rats which were given the estrogen together with thiouracil for 21 days. However, when rats received thiouracil alone for a 2-week preliminary period, the subsequent administration of estrogen plus thiouracil for 10 days failed to maintain even the normal level of lactogenic hormone.

The pituitary and thyroid weights of the thiouracil as well as thiouracil plus estrogen-treated rats were increased above the normal controls. The thyroid hypertrophy in the rats which received estrogen and thiouracil for 10 days following a preliminary treatment of thiouracil alone for 2 weeks was less than in the rats which received only thiouracil for the same period.

15839

A Brief Insulin Tolerance Test.*

G. J. KRONENBERG AND W. S. McCULLOCH.†

From Department of Psychiatry, University of Illinois College of Medicine, Chicago, Ill.

Meduna *et al.*¹⁻³ by determining reducing substance (which is predominantly glucose) in venous blood at fasting and at 5, 10, 15, 20, 25, 30, 35, 45, 60, 90 and 120 minutes following the injection of 0.1 unit insulin per kg body weight, had found that the nadir was shallower and later in oneirophrenics than in normals, and had observed, in individual, normal records, a bump—i.e. a failure to fall or actual rise—before the nadir at which sympathetic counter-regulation initiates a

rapid rise in blood-sugar peaking about the 45th minute. He had also found that throughout all of these changes the amount of reducing substances other than glucose was constant. The present work was begun in 1943 to increase the certainty of his separation and, by samples at the 12th and 17th minutes, to define the bump on the descending limb and finally, to construct a brief test for clinical use.

On the assumption that the effect of insulin in lowering the blood-sugar was proportional to its initial value, Meduna had expressed the observed decrease in percent of fasting value, and subsequent experience indicates that this is probably the best expression. He used colorimetric determination of alkaline ferricyanide reduced by tungstic acid filtrates from 0.1 ml of venous blood and this, by chance, gives mean fasting values of approximately 100 mg %, so that the mean

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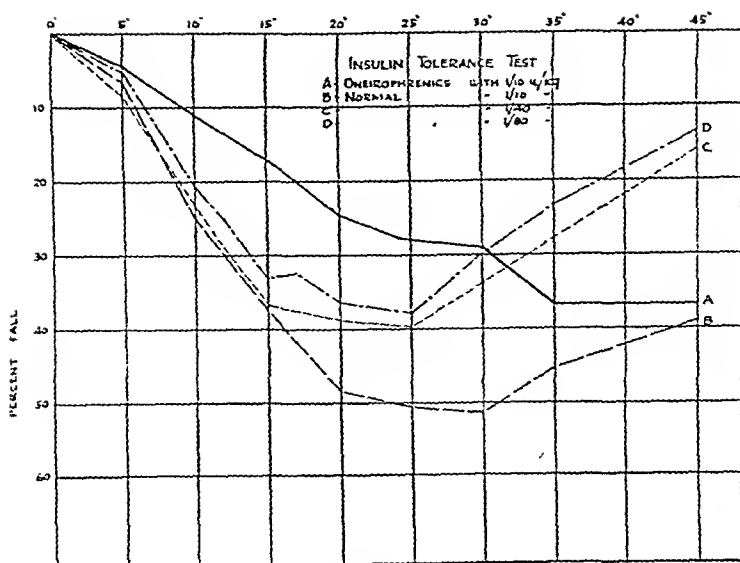


FIG. 1.

Insulin Tolerance Test in Oneiophrenics and Normals. Mean % fall of reducing substance of the blood in oneiophrenics (A) and in normals, showing early divergence and normal lack of dose sensitivity for the first 15 minutes, with doses of (B) 1/10, (C) 1/40, and (D) 1/80 u/kg.

TABLE I.

Subject	Dose	15'	20'	25'	30'
A Oneiophrenic	1/10 u/kg	16.8 σ 7.6	24.3 σ 9.8	27.6 σ 4.4	29.8 σ 8.9
B Normals	1/10 "	37.6 σ 10.1	48.4 σ 9.5	50.6 σ 8.8	51.7 σ 9.2
C "	1/40 "	36.8 σ 9.3	38.8 σ 9.0	39.8 σ 9.0	33.5 σ 9.6
D "	1/80 "	33.0 σ 7.0	36.3 σ 7.8	37.8 σ 7.6	29.0 σ 6.9
AB	Fisher's t	7.75	8.2	7.0	8.1
AC	and prob.	7.5	<.01	<.01	<.01
AD		6.87	<.01		

fall in percent of fasting is approximately equal to the mean fall in mg %. The present data was obtained and charted by his methods.

The following curves are based on the first 20 to 25 cases in which the diagnosis of the patient was certain, the health of the control assured, the technic faultless and the data available for computation at the time this material was organized. cursory examination of more than 300 records has indicated no important exceptions.

Fig. 1A is based on Meduna's data and shows the mean blood-sugar level at various times during the first 45 minutes after injection of 0.1 unit insulin per kg body weight in his first 21 reported tests of oneiophrenics. Fig. 1, B, C, and D are the same for healthy

controls receiving that standard dose and $\frac{1}{4}$ and $\frac{1}{8}$ of it respectively. These 3 normal curves are similar for the first 15 minutes and all fall far faster than the oneiophrenic at that time.

Table I gives the mean falls, the standard deviations, Fisher's "t" and the probability that the difference between the means is due to chance at the 15th, 20th, 25th and 30th minutes. From this it is clear that oneiophrenics differ significantly from normals through the 30th minute after which normals rise while some oneiophrenics continue to descend.

Table I also shows the lack of any reliable difference between the 3 groups of controls during the first 15 minutes, indicating that the rate of fall is independent of the dose

BRIEF INSULIN TOLERANCE TEST

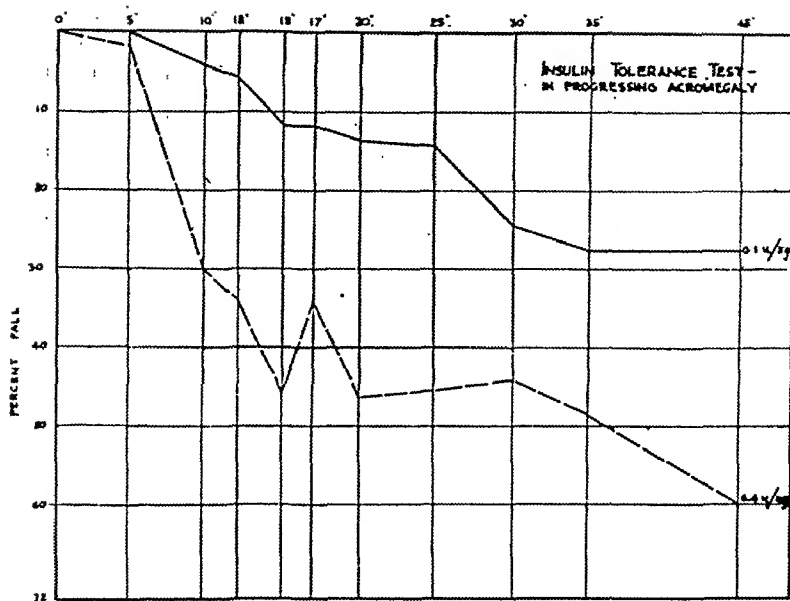


Fig. 2.

Dose Sensitivity in Acromegaly. Insulin tolerance curves in % fall of reducing substances of blood after 0.1 u/kg showing resistance but rapid fall and spike on descending limb after 0.4 u/kg.

which yet determines the nadir.

Finally, Table I shows that at the 15th minute the differences between oneirophrenics and normals receiving $\frac{1}{2}$ as much insulin is still significant. In a few cases we have given 8 times the dose to oneirophrenics and found that, though it made the blood-sugar fall farther, it fell no faster. Obviously, therefore, in a clinical test one should use the early part of the test where a gross error in dose is insignificant.

Of many nondiabetic, medical and surgical conditions tested for increased resistance to insulin only one group of malignant hypertensives and the progressive acromegalic have shown resistance. The latter (Fig. 2) are interesting because the resistance can be exceeded by increasing the dose and then there may appear an exaggeration of the bump on the descending limb. Similar exaggerations have appeared following sundry operations on the brain.

The time at which this upward inflection occurs varies from the 12th to the 17th minute. A third of the bumps are actual spikes, a third are levels and a third are only decreased rates before the final, fast drop to

the nadir. It is absent in less than one-tenth of normals and one-third of oneirophrenics, but it is frequently missed with 5-minute sampling and its temporal scatter averages it out in the mean curve except with the smallest dose. The mean value of the difference between the preceding and subsequent low and the mean highest value of the bump exceeds twice the mean error of the method and hence the probability that the bump is due to chance is negligible.

It is an increment in glucose. It is present in arterial blood. It occurs with all varieties of American insulin and with Danish insulin having no hyperglycemic factor. It is present in diabetes mellitus, Addison's disease, malignant hypertension, and after sundry insults to the brain. It is not prevented by terror or atropine, by sympathectomy or supradiaphragmatic vagotomy, by advanced disease of the liver or by splenectomy. It is not preceded or accompanied by any signs of autonomic change in EEG, EKG, blood pressure, respiration, pupillary diameter or dorsal skin potential. Presumably it is due to some as yet undiscovered mechanism counteracting some effect of insulin. Fortunately

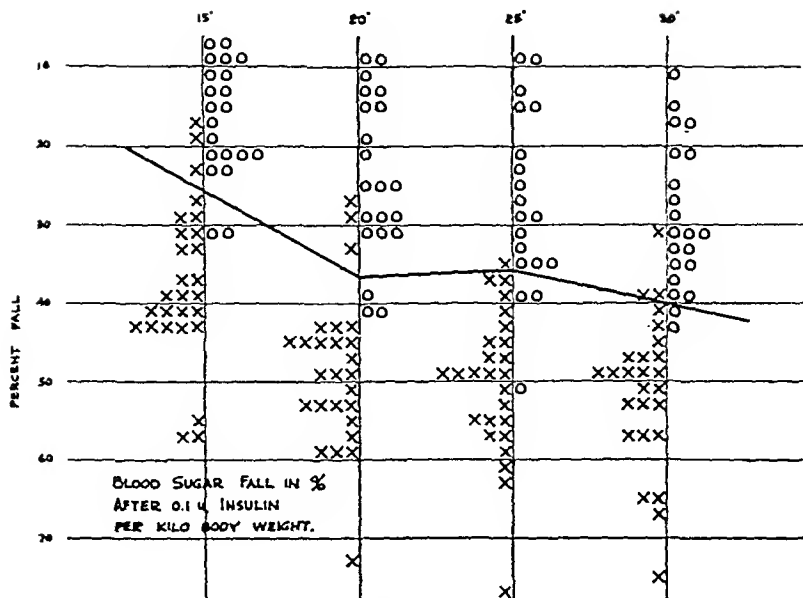


FIG. 3.

Scattergram for Use with Brief Insulin Tolerance Test. Scattergram of oneirophrenic, O, and normal, X, % fall from fasting of reducing substances of the blood.

it is not violent enough to prevent separation of oneirophrenics from normals.

Although in a given normal the blood-sugar may fall as fast and as far with an 80th as with a 10th unit insulin per kg body weight, the signs of so-called hypoglycemic shock—faintness, trembling, sweating, cardiac acceleration, and pupillary dilation—are minimal or absent. This would incline one to use the smaller dose, but as the law forbids sale of insulin at less than 20 units per cc one has to make up fresh sterile solutions for measurable doses, and many neurotics are resistant to it though they react normally to the regular dose. As the separation between oneirophrenics and normals is equally certain from the 15th through the 30th minute, only time is conserved by early sampling; but at the 15th minute the blood-sugar is falling 5 mg per minute in the normal which means that the timing must be accurate and venous return unobstructed. It is easier to note the exact time at which a sample is drawn than to draw it at a prescribed moment.

The following procedure, therefore, is recommended. The fasting sample is taken, then 0.1 unit insulin per kg body weight is

injected intravenously. One or two samples are then taken between the 15th and 30th minutes and the time noted accurately. The patient may then be given sugar if desired. The total reducing substance is determined in each sample as noted above. If the fasting value differs significantly from 100 mg %, the falls are expressed in per cent of fasting; if not, they are left as mg %. Any such fall from fasting and the time after injection of insulin can then be compared with Fig. 3. This is a scattergram showing the distribution of oneirophrenics and normals on which is drawn one line running through those points at which a given value is as likely to be found in an oneirophrenic as in a normal. Inasmuch as the overlapping of the scattergrams in the beginning and end of this interval, and no individual curves have been found to have more than one point across the line, 2 samples on either side of the line enhance the certainty of diagnosis. The chance of an erroneous diagnosis is, in fact, less than the product of chances for the 2 separately.

Summary. For the first 15 minutes the rate of fall in reducing substances in the blood

expressed in per cent of fasting value is not much affected by decreasing the size of the intravenous dose of insulin below 0.1 u/kg or by increasing it in oneirophrenics. At 15 minutes the separation is statistically valid despite a bump of unknown significance on

this descending limb of the curve. Therefore, it is possible, in patients suspected of oneirophrenia, to estimate the resistance to insulin with a statistical validity as great as can be obtained from much longer tests.

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Thiouracil in the Treatment of Leukemia.

O. C. HANSEN-PRUSS. (Introduced by W. A. Perlzweig.)

From the Department of Medicine, Duke University Medical School.

It has been observed that patients with leukemia may show many of the clinical features of hyperthyroidism, such as nervousness, tachycardia, decreased cholesterol in the blood, and increased metabolic rate. Temporary improvement may result from the rational use of Lugol's solution.¹⁻³

During the past 15 years, 175 patients with various types of leukemia have been studied in this clinic. The disease remained stationary or progressed slowly as long as the cholesterol level was above 150 mg %, but progressed rapidly when the cholesterol values fell below 100 mg %.

The cholesterol levels appeared to be influenced by the absolute number of extremely immature forms and not by the total number of circulating leukocytes. The cholesterol levels decreased as the number of immature forms increased, but in one group of 15 patients who progressed rapidly to a fatal end, with extensive systemic and visceral myeloblastic proliferation, the total counts were slightly elevated, normal or reduced, and the absolute number of myeloblasts small, yet the cholesterol levels were constantly below 100 mg %.

In remissions induced by X-ray therapy, the serum cholesterol values were uniformly

above 150 mg %, and as the patients became resistant to the X-ray, the cholesterol levels fell.

There was no constant relationship between the basal metabolic rate, the serum cholesterol values and the prognosis.

It seemed desirable to determine whether the administration of thiouracil would increase the serum cholesterol levels and improve the patient's symptoms.

Case 1. S.K., white female, age 29 years with chronic myelogenous leukemia. The disease was apparently in its second year when it was recognized in 1940. Between October 1940 and June 1945, she received several series of X-ray treatments. These were given by spray and locally over the spleen. (These treatments totalled 1100 R. by spray and 1950 R. over the spleen). She experienced several striking clinical and hematological remissions. The last X-ray treatment was on June 5, 1945. The subsequent events are summarized in Table I.

This patient received 1.5 g of thiouracil a day over a period of 60 days, or a total of 90 g. She tolerated the drug quite well. She had an irregular, intermittent fever which was present before the administration of thiouracil was begun and persisted thereafter. At first, she seemed clinically improved; she felt stronger and the hemoglobin and red cell levels rose temporarily. The circulating leucocyte count fell precipitately during the first 24 hours after the drug was given, but

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TABLE I.
Temporary Drop in the White Count After Thiouracil in Chronic Myelogenous Leukemia.

Date	Thiouracil g/day	WBC (thous.)	Mature Myeloid G. %	Hgb., g	RBC (mill.)	B.M.R. %	Serum cholest., mg %	Bone marrow		
								WBC (thous.)	Myelocytes, %	Myeloblasts, %
9/10/45	—	400.0	45	6.0	3.0	+14	165	274	33	17
11	1.5	*430.0	—	6.4	2.0	—	—	—	—	—
12	"	*109.0	50	6.3	—	—	—	—	—	—
13	"	130.0	—	—	—	—	—	—	—	—
14	"	134.0	59	7.2	3.1	—	175	280	42	22
21	"	170.0	60	7.3	2.3	+17	210	—	—	—
28	"	155.0	39	8.3	2.5	+24	220	—	—	—
10/ 3	"	146.0	45	8.6	2.5	+8	210	—	—	—
10/ 8	"	130.0	40	10.6	3.5	+10	210	—	—	—
11/12	"	283.0	32	7.3	2.3	+14	145	450	28	46

* Checked repeatedly.

there was no corresponding quantitative change in the bone marrow. Furthermore, there was no appreciable change in the maturation pace of the bone marrow elements. The drug did not affect decisively the red cells, hemoglobin or platelets. The basal metabolic rate did not fall to any important extent and the cholesterol level did not change. She grew gradually worse, lost weight, became steadily weaker and the spleen enlarged progressively and rapidly. In mid-November, she developed purpura (platelet count 100,000). It is obvious that thiouracil had failed to decrease the myeloid hyperfunction and leukocytosis and to influence the rate and degree of maturation of the myeloid elements. The patient became resistant to X-ray therapy, failed rapidly and died 7 months later.

Case 2. D.C.L., white female, age 49 years. Chronic myelogenous leukemia diagnosed in 1941. Between July 1941 and July 1945 she received a series of X-ray treatments by spray and over the spleen. (A total of 1150 R. over the spleen and 550 R. by spray). The induced hematological and clinical remissions were satisfactory. The last X-ray treatment before thiouracil was given on January 11, 1945. The subsequent developments are shown in Table II.

This patient received a total of 26.7 g of thiouracil in 15 days. She tolerated the drug quite well and remained afebrile. There was no significant quantitative or qualitative change in the peripheral white blood cell picture or in the bone marrow pattern. The red cell count and hemoglobin value remained unchanged throughout the period of observation. There was no demonstrable increase or depression of red cell formation, nor the platelets influenced. It is apparent that thiouracil, in this patient, failed to depress the basal metabolic rate since at the end of the second week the basal metabolic rate had risen to +47% and the serum cholesterol had fallen materially. A partial remission in the disease was induced by X-ray treatments later on.

Case 3. B.E.D., white male, age 27 years, suffering from myelogenous leukemia of 3

TABLE II.
Negative Effect of Thiouracil on Total White Count in Myelogenous Leukemia.

Date	Thiouracil g/day	WBC (thous.)	Mature Myeloid C. %	Immature Myeloid C. %	Hgb., g	RBC (mill.)	B.M.R. %	Serum cholest., mg %	Bone marrow		
									WBC (thous.)	Myelocytes, %	Myeloblasts, %
7/ 3/45	Received 3 blood transfusions	500	38	47	6.4	2.0	+31	205	458	32	6
9	—	346	67	26	8.0	2.2	—	—	—	—	—
10	1.6	520	50	49	9.0	2.9	—	200	—	—	—
12	"	515	52	45	9.8	2.9	—	—	—	—	—
15	"	370	36	62	9.7	2.7	—	—	—	—	—
16	1.9	445	37	63	8.8	3.1	+32	205	—	—	—
17	"	378	43	56	9.7	3.3	+47	120	383	50	7
24	"	85	81	17	9.1	3.1	—	200	—	—	—
25	X-ray begun										
8/ 1											

TABLE III.
Striking Failure of Thiouracil in Lowering the Basal Metabolic Rate in Chronic Myelogenous Leukemia.

Date	Thiouracil g/day	WBC (thous.)	Mature Myeloid C. %	Immature Myeloid C. %	Hgb., g	RBC (mill.)	B.M.R. %	Serum cholest., mg %	Bone marrow		
									WBC (thous.)	Myelocytes, %	Myeloblasts, %
8/ 6/45	—	56.0	*61	36	6	1.8	+70	83	250	40	15
7	1.6										
9	"	26.2	*58	32	6	2.0	—	—	—	—	—
13	"	30.0	*63	20	6.9	1.9	+89	70	—	—	—
19	"	37.5	*47	41	6.8	1.8	—	—	215	†38	28

* 25% or more were basophiles.

† Many basophiles.

years duration. He had received a series of X-ray treatments with temporary improvement. The last X-ray treatment was given in December 1944, 8 months before thiouracil was tried. (A total of 1350 R. by spray and 1075 R. over the spleen). The result of thiouracil treatment is summarized in Table III.

It is obvious that thiouracil did not affect the total circulating white cell mass, the bone marrow pattern, the basal metabolic rate or the serum cholesterol. The erythroblastic system was not influenced. This patient's high basophile count was probably due to the prolonged X-ray treatments. Thiouracil, again, did not influence the hemoglobin, red cell or platelet values. This patient expired two weeks later with profuse internal and external hemorrhages.

Case 4. E.M.G., white female, age 40 years. This patient had noticed repeated bleeding into the skin, from the gums, and a progressive abdominal discomfort over a period of one year. She was admitted to the surgical service and the disease was recognized as chronic myelogenous leukemia with striking splenomegaly. She received a series of X-ray treatments by the spray method and over the spleen. (Spray 100 R.; spleen 450 R.) Since she had not received any previous treatments, the patient was considered a suitable subject to test the effect of thiouracil on the X-ray-induced remission in myelogenous leukemia. These observations are shown in Table IV.

It is apparent that thiouracil did not prolong or modify the X-ray-induced remission. She received 359.3 g of the drug in 10 months. The variations in the metabolic rate and serum cholesterol are rather striking, but inconclusive.

Two other patients with chronic myelogenous leukemia were given thiouracil to test the effect of this drug on X-ray-induced remissions. The results were analogous to that described above. It is obvious that thiouracil does not prolong or modify the remissions induced by X-ray treatments.

Thiouracil was given to one patient with an untreated myeloblastic leukemia. He re-

TABLE IV.
Effect of Thiouracil on the Duration of X-Ray Induced Remission in Chronic Myelogenous Leukemia.

[illegible]

ceived 1.5 g a day. There was no change in the peripheral white cell picture, qualitatively or quantitatively, nor did the bone marrow pattern change. This patient expired on the 15th hospital day of thiouracil treatment.

Discussion and Conclusions. Thiouracil was administered to 6 patients with chronic myelogenous leukemia, and to one patient with an acute myeloblastic leukemia. The dosage averaged 1.5 g a day and was well tolerated by every patient. One patient (case 4) received a total of 359.3 g of the drug over a 10-month period. The drug had no effect on the total circulating white count, nor on the differential white cell picture, red cell elements, hemoglobin or platelet values or the bone marrow pattern.

Recently, and after completion of this study, one observer reported that he had tried thiouracil in doses of 3 mg daily in a patient with myelogenous leukemia without appreciable results.⁴

⁴Limarzi, L. R., Kulasavage, R. J., and Pirani, C. L., *J. Hem. (Blood)*, 1946, 1, 426.

Thiouracil did not influence the metabolic rate of leukemic patients materially when given in a dose of 1.5 g daily. This was true whether it was administered over a period of 2 weeks, 2 months or 10 months. The serum cholesterol rose in only 2 patients (cases 2 and 4), and this rise was only transitory. In one patient (case 3) the serum cholesterol continued to fall, the basal rate steadily rose despite the administration of thiouracil. In one patient reported (case 4), and in 2 others, this drug failed to prolong or modify the remission induced by irradiation. Thiouracil given in the dosage reported in this series had no demonstrable effect on the myeloid dysfunction, basal metabolic rate, the serum cholesterol level or the bone marrow pattern of patients with myelogenous leukemia.

Thiouracil, furthermore, did not modify the clinical course of the disease. It seems certain therefore, that this drug has no value as an adjunct in the treatment of myelogenous leukemia.

15841

Effects of Liver Feeding on Growth and Ovarian Development in the Hyperthyroid Rat.*

BENJAMIN H. ERSHOFF.

From the Emory W. Thurston Laboratories, Los Angeles, California.

It has been demonstrated that excessive doses of thyroxin or desiccated thyroid cause anestrus and loss of body and ovarian weight in the adult rat and prevent gonadal development in the young animal. The deleterious effects in the adult rat according to Drill

*et al.*¹ can be prevented by the administration of increased amounts of thiamine or yeast. Such nutrients are without effect, however, in the young animal.² It appears, therefore, either (1) that the young rat is more susceptible to the toxic effects of thyroid insofar as gonadal physiology is concerned or (2) that excessive thyroid feeding may precipitate a deficiency of some nutrient other than the above necessary for gonadal development in the immature rat. In the

*The subject matter of this paper has been undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The opinions or conclusions contained in this report are those of the author. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.

¹ Drill, V. A., Overman, R., and Leathem, J. H., *Endocrinology*, 1943, 32, 327.

² Ershoff, B. H., *Endocrinology*, 1945, 37, 218.

present experiment attempts were made to prevent by dietary means the failure of ovarian development in the immature thyroid-fed rat.

Procedure and Results. Three basal rations were employed in the present experiment: diets A, B and C. Diet A was a purified ration containing the B complex factors in synthetic form only. Diets B and C were similar in composition but contained yeast or desiccated liver in addition to the synthetic vitamins. All 3 rations were supplemented with 0.0 and 0.5% U.S.P. desiccated thyroid. Seventy-eight female rats of the Long-Evans strain were weaned at 21 to 23 days of age and litter mates divided as far as possible among the experimental groups listed in Table I. Animals were kept in metal cages with raised screen bottoms to prevent access to feces, and sufficient food was administered to assure *ad lib* feeding. Subsequent to the 40th day of feeding, vaginal smears were taken daily of all rats. Surviving animals were autopsied on the 60th day of feeding; ovaries were weighed and fixed in 10% formol, and sections prepared and stained with hematoxylin and eosin.

Results are summarized in Table II. In agreement with earlier findings¹ the mortality rate of thyroid-fed rats was greater on the synthetic diet than on rations containing yeast. Diets containing liver were similarly effective in prolonging survival. A significant difference in body and gonadal weight was observed in thyroid-fed rats on the various diets employed. On diets A and B both body and ovarian weight was significantly less in thyroid-fed rats than in animals fed a similar diet with thyroid omitted; and ovaries of the thyroid-fed animals resembled in microscopic appearance those of the immature rat. On diet C, however, no significant differences in body and gonadal weight were observed between animals fed thyroid and those on a thyroid-free ration; and histologically the ovaries of thyroid-fed rats appeared normal in all respects. In the absence of dietary thyroid body and ovarian weights did not differ significantly on any of the diets employed; and histologically ovaries appeared normal in all groups. Data

TABLE I.
Composition of Experimental Diets.

Dietary component	Diet A	Diet B	Diet C
Yeast*	0.0	10.0	0.0
Liver†	0.0	0.0	10.0
Vitamin Test Casein‡	22.0	22.0	22.0
Salt Mixture§	4.5	4.5	4.5
Sucrose	73.5	63.5	63.5

When thyroid was fed it was included in the above diets at a level of 0.5%, replacing an equal amount of sucrose.¶

To each kg of the above diets were added the following synthetic vitamins: thiamine hydrochloride 72 mg, riboflavin 9 mg, pyridoxine hydrochloride 15 mg, calcium pantothenate 67.2 mg, nicotinic acid 60 mg, 2-methyl-naphthaquinone 5 mg, and choline chloride 1.2 g. Each rat also received 3 times weekly the following supplement: cottonseed oil (Wesson) 500 mg, alpha-tocopherol 1 mg, and a vitamin A-D concentrate[§] containing 50 U.S.P. units of vitamin A and 5 U.S.P. units of vitamin D.

* Brewer's Type Yeast No. 200, Anheuser-Busch, Inc., St. Louis, Mo.

† Whole Dried Liver Powder, Armour and Co., Chicago, Ill.

‡ Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

§ Sure's Salt Mixture No. 1.3

¶ Thyroid Powder, U.S.P., Armour and Co., Chicago, Ill.

§ Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per gram.

obtained from vaginal smears check with the above findings. On diets A and B thyroid-fed rats exhibited constant leucocytic smears subsequent to the 40th day of feeding in contrast to the regular estrus cycles obtained on diet C. In the absence of dietary thyroid estrus cycles were regular on all diets. Subsequent work indicates that the ovaries of thyroid-fed rats raised to maturity on diet C were normal functionally as well as histologically. When bred to normal males after the 60th day of feeding, such animals while still on the same diet gave birth to apparently normal young.

Available data indicate that the factor in liver responsible for the above effects is not one of the major B vitamins. Doubling the B vitamin content of diet A was without significant effect on the body or gonadal weight of thyroid-fed rats. Individual supplements

³ Sure, B., *J. Nutrition*, 1941, **22**, 499.

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Procedure and Results. Three basal rations were employed in the present experiment: diets A, B and C. Diet A was a purified ration containing the B complex factors in synthetic form only. Diets B and C were similar in composition but contained yeast or desiccated liver in addition to the synthetic vitamins. All 3 rations were supplemented with 0.0 and 0.5% U.S.P. desiccated thyroid. Seventy-eight female rats of the Long-Evans strain were weaned at 21 to 23 days of age and litter mates divided as far as possible among the experimental groups listed in Table I. Animals were kept in metal cages with raised screen bottoms to prevent access to feces, and sufficient food was administered to assure *ad lib* feeding. Subsequent to the 40th day of feeding, vaginal smears were taken daily of all rats. Surviving animals were autopsied on the 60th day of feeding; ovaries were weighed and fixed in 10% formol, and sections prepared and stained with hematoxylin and eosin.

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§ Sure's Salt Mixture No. 13

¶ Thyroid Powder, U.S.P., Armour and Co., Chicago, Ill.

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TABLE II.
Effects of Thyroid Feeding on Body and Gonadal Weight.

Dietary group	% thyroid	No. of animals	Avg body wt		Avg ovarian wt* mg
			Initial g	On 60th feeding day* g	
A	.5	20	43.7	116.5 ± 6.7 (4)	23.6 ± 2.2
B	.5	20	42.9	133.5 ± 7.8 (14)	27.8 ± 4.1
C	.5	20	43.3	202.3 ± 8.1 (15)	72.3 ± 7.1
A	.0	6	42.7	198.7 ± 4.1 (6)	53.1 ± 3.3
B	.0	6	43.1	186.6 ± 6.7 (6)	56.8 ± 4.7
C	.0	6	42.7	197.5 ± 4.4 (6)	59.4 ± 5.1

The values in parentheses indicate the number of animals which survived of which this is an average.

* Including standard error of the mean calculated as follows:

$$\sqrt{\frac{ed^2}{n}} / \sqrt{n}$$

where "d" is the deviation from the mean and "n" is the number of observations.

of thiamine, riboflavin, pyridoxine, calcium pantothenate, nicotinic acid, inositol, *p*-aminobenzoic acid, biotin or folic acid were similarly ineffective.[†] Doubling the salt content of diet A or administering an additional 10% casein or 10% wheat germ[‡] at the expense of an equal amount of sucrose were also without effect. Only liver of all substances tested gave a positive response in the thyroid-fed rat. Preliminary results indicate that

† Supplements were added to diet A in the following amounts per kg of diet: thiamine hydrochloride 3.6 g, riboflavin 450 mg, pyridoxine hydrochloride 750 mg, calcium pantothenate 3.36 g, nicotinic acid 3.0 g, inositol 10.0 g, *p*-aminobenzoic acid 10.0 g, biotin 1.0 mg, and folic acid 10.0 mg. We are indebted to Dr. Stanton M. Hardy of the Lederle Laboratories, Pearl River, New York, for the folic acid employed in the present experiment. The remaining B vitamins were obtained from Merek and Co., Rahway, N.J.

Subsequent work indicates that individual supplements of ascorbic acid and the fat-soluble vitamins were also ineffective. These were added to diet A in the following amounts per kg of diet: ascorbic acid 10 g, vitamin A 100,000 U.S.P. units, vitamin D 25,000 U.S.P. units, and 2-methyl-naphthaquinone 100 mg. Alpha-tocopherol was administered as a daily supplement at a level of 5 mg per rat.

‡ Defatted Wheat Germ, Vio-bin Corporation, Monticello, Ill.

the factor in liver responsible for the above effects is heat stable.

Discussion. Evidence is accumulating that in addition to the major nutrients, substances are present in our diet which may be required in increased quantities during conditions of stress. Such factors are apparently dispensable under normal conditions or their requirements are so small they may readily be met by amounts present in the diet or through the synthetic activity of the intestinal flora or the animals' own tissues. Certain drugs or other "stress factors" may, however, increase requirements for these substances to the extent that deficiencies occur, manifest by retarded growth or tissue pathology, and preventable by the administration in appropriate amounts of the missing nutrient. Present findings indicate that thyroid feeding is such a factor. Although liver did not counteract other manifestations of hyperthyroidism, such as a hypertrophied ventricular wall, reduction in the creatine content of the heart or an increased B.M.R.,⁵ it did counteract completely the retardation of growth and inhibition of ovarian development observed on all other rations when thyroid was fed.

Findings indicate that liver contains an or-

ganic factor other than thiamine, riboflavin, pyridoxine, calcium pantothenate, nicotinic acid, inositol, *p*-aminobenzoic acid, biotin or folic acid, a substance not present in significant amounts in wheat germ, casein or yeast, but required for normal growth and ovarian development in the immature thyroid-fed rat.

Summary. The administration of liver completely counteracted the retardation of growth and inhibition of ovarian development

observed in immature rats fed toxic amounts of thyroid. Wheat germ, yeast, or increased amounts of salt mixture, casein, thiamine, riboflavin, pyridoxine, calcium pantothenate, nicotinic acid, inositol, *p*-aminobenzoic acid, biotin or folic acid were ineffective in this regard. The suggestion is made that liver contains some factor other than the above required for normal growth and ovarian development in the immature thyroid-fed rat.

15842

Some Pharmacological Characteristics of Bacitracin.*

JOHN V. SCUDI AND WILLIAM ANTOPOL.

From the Department of Pharmacology, College of Physicians and Surgeons, Columbia University, New York, and the Division of Laboratories, Newark Beth Israel Hospital, Newark, N. J.

Bacitracin, an antibiotic discovered by Meleney and co-workers¹ in the filtrate of surface cultures of the Tracy strain of *B. subtilis*, has become of increasing interest, particularly because of its potential value in the treatment of penicillin-resistant infections. Because of this increased interest, the following preliminary data on the toxicological and pharmacological properties of the antibiotic are presented.

Acute toxicity experiments. Fresh bacitracin[†] solutions used for oral and intraperitoneal administration contained the dose in 1 cc of water; solutions for intravenous and subcutaneous injections contained the dose in 0.25 to 0.50 cc per 20 g of mouse, or 50 g of rat. All samples gave clear solutions at pH 6 to 7 except No. 187, which dissolved at pH values of 5 to 6. For oral or subcutaneous administration, fine suspensions

were administered because of the large doses required. Solutions of earlier samples of bacitracin were brown, but more highly purified later samples, illustrated by No. B-100, were almost colorless.

Experiments were performed with male, white, Rockland Swiss mice weighing 20 to 25 g, and male white rats of the Sherman strain maintained on a stock diet with free access to food and water at all times. Surviving animals were observed for a period of at least 7 days after acute or chronic dosing.

Results of the acute toxicity experiments are shown in Table I. Comparison of the first 4 samples, all administered intraperitoneally, indicates that the toxicity varies independently of the activity. (The fourth sample, No. B-100, is much less toxic than the other 3 and is representative of the batches now being produced). In this connection it may be noted that inactivation of the first sample under mild conditions (pH 7.0 at 37° for 8 days) did not alter the toxicity of the preparation. The LD₅₀, 200 mg per kg, did not differ significantly from the value of 240 mg per kg obtained with the active sample. Neither the scattering of the deaths, nor the toxic signs, nor the pathological lesions in mice differed appreciably

* The work described in this paper was done under a contract between the Office of the Surgeon General and Columbia University. Administration of the contract was directed by Dr. Frank L. Meleney.

¹ Johnson, B. A., Anker, H., and Meleney, F. L., *Science*, 1945, 102, 376.

[†] We are indebted to Dr. John T. Goorley of the Ben Venue Laboratories for the bacitracin.

TABLE I.
Acute Toxicity of Bacitracin in Mice and Rats.

Sample No.	Animal	Mode of administration	Activity of sample U per mg	No. of animals between LD ₁₆ and LD ₈₄	LD ₅₀ mg per kg	LD ₅₀ units per kg	σ	Stand. dev. LD ₅₀
1	187	I†	35	170	240	8,500	3,800	410
2	188	"	13	90	650	8,500	2,000	300
3	203	"	21	113	360	7,500	2,400	320
4	B-100 new process	"	30	80	500	15,000	4,900	770
5	187	"						
	inactivated	"	0	40	200			
					$\sigma = 50$			
6	203	Iv§	21	75	SD.LD ₅₀ = 11	7,500	1,300	210
7	203	S	21	75	2,500	52,000	20,000	3,300
8	208WI	"	24	70	1,300	31,000	7,000	1,200
9	187	I†	35	49	190	6,700	2,500	500

* The majority of deaths occurred during the first 24 hours, with large numbers dying during the second 24 hours, and occasional deaths thereafter.
† All deaths occurred within the first 24 hours.
‡ I = intraperitoneal.
§ I = intravenous.
|| S = subcutaneous.

from those observed with the active material. Since the toxicity of the sample was not altered by destruction of the active principle, it seems reasonable to assume that further purification of bacitracin will lead to a lowered toxicity. The third sample (203) gave identical LD₅₀'s after intravenous and intraperitoneal injections. The acute subcutaneous and oral toxicities of bacitracin were much lower than the intraperitoneal toxicity: for example, sample No. 203 was about 7 times as toxic when given intraperitoneally as when given subcutaneously. There was no direct comparison for the eighth sample (208 WI) but here again the subcutaneous toxicity represented by the LD₅₀ was notably low. Given orally in amounts increasing up to 3.75 g per kg or 131,000 units per kg, sample No. 187 failed to produce death. At 7.5 g per kg 2 mice out of 5 died. These deaths may have been influenced by the high osmotic concentration of the solutions administered. The oral and subcutaneous findings are similar to those observed by Molitor *et al.*² in a study of streptomycin.

The acute intraperitoneal toxicity of sample 187 was significantly greater in rats than in mice. There were no deaths subsequent to the first 24 hours after the administration of the drug to rats. Further, renal lesions, described below, were striking in mice but were minimal in rats. The intravenous toxicity of bacitracin appeared to be greater in rats than in mice. Sudden, convulsive deaths could be produced regularly at doses as low as 4000 units per kg of sample No. 187 intravenously.

Signs of acute toxicity were similar regardless of the sample of bacitracin used. Following intraperitoneal injection of lethal doses, mice exhibited hyperirritability and clonic convulsions followed by an increasingly severe depression. Central respiratory paralysis appeared to be the cause of death in both mice and rats. Similar effects were produced more rapidly by intravenous injections. No acute toxic effect other than

² Molitor, H., Graessle, O. E., Kuna, S., Mushett, C. W., and Silber, R. H., *J. Pharm. Exp. Ther.*, 1946, 86, 151.

apathy was noted after oral or subcutaneous administration of the impure antibiotic. Complete blood counts were made in all mice prior to, and serially in surviving mice for 8 days after the intraperitoneal injection of B-100. Although large doses, grouped around the LD₅₀, were given, no abnormalities in blood morphology were observed.

Chronic toxicity experiments. Groups of 25 mice were given bacitracin (No. 187) subcutaneously at doses of 2, 20, and 50 units per 20 g mouse† every 4 hours for 5 days. The total dose administered during the 5-day period was 3,000, 30,000 and 75,000 units per kg of mouse. None of the animals died during the period of treatment nor in the following 7 days. The animals appeared normal and made average weight gains of 5% of body weight during the 5 days of treatment and 10% during the ensuing 7 days, regardless of the dose given.

Rats weighing 80 to 90 g were given 6,000 units per kg of bacitracin intraperitoneally. One week later surviving animals were divided into 2 groups of 10 each. Animals in the first group were given, subcutaneously, 650 units per kg per day, 5 days of each week for 13 doses followed by 12 additional doses intraperitoneally. Animals in the second group were maintained on the same schedule but the chronic doses were doubled. Rats in the first and second groups received a total of 22,250 and 38,500 units of bacitracin per kg, respectively. With the exception of local areas of alopecia at the site of injection all animals appeared grossly normal. During the 5 weeks of treatment, animals of the first group grew from an average of 88 to 177 g. Those in the second group grew from 84 to 150 g, suggesting that the larger doses impeded growth to a slight extent.

Pathological changes in mice and rats following single or repeated doses. Gross examination of the organs following acute deaths showed congested splanchnic vessels and emphysematous lungs. Mice which survived

large doses for more than 24 hours lost weight and exhibited enlarged, edematous, ischemic kidneys. Kidney involvement appeared maximal 3 to 5 days after intraperitoneal injection of bacitracin, and then appeared to regress. With the exception of adrenal enlargement, remaining organs were normal grossly. At the site of subcutaneous injection, induration, congestion and hemorrhage were often found with the early preparations; product B-100, manufactured by a new process, caused considerably less irritation.

Histologically, renal damage in the mouse was striking. With moderate involvement, the cytoplasm of cells of the convoluted tubules was filled with fine eosinophilic granules, particularly in the free portion of the cell; in places it appeared as though this portion of the cell was being cast into the lumen. The loops of Henle, particularly the descending limbs, were filled with deeply staining eosinophilic material usually in the form of casts or globules. Similar material was also present in the collecting tubules. The loops of Henle and collecting tubules were markedly dilated. With severe involvement there was tubular necrosis so that identification of the individual tubule was difficult. There was necrosis of the convoluted tubules which appeared to involve the distal convoluted tubules and, occasionally, the proximal tubules. The lumina were filled with necrotic cells, nuclear debris and calcium particles. Similar material was found in the loops of Henle and collecting tubules. Rarely a tubule was calcified. Bowman's space was distended with coagulum but the glomerular tuft proper appeared uninvolved. Mice in which appreciable tubular necrosis was found were either dying or were sacrificed when moribund.

Mice which survived 6 days after administration of the drug showed marked dilation of the collecting tubules and, to a less extent, the loops of Henle. The collecting tubules contained eosinophilic globoid bodies or casts which completely filled the lumen and had the appearance of coagulated serum. At times basophilic bodies were found. Tubular calcification was not frequent. After 10 to

† This may be assumed from the work of Meleney *et al.* to represent 1, 10, and 25 times the therapeutic dose.

12 days a less pronounced picture was observed. There were small depressed subcapsular areas composed of collapsed tubules, and a rare atrophic glomerulus. Cystically dilated tubules and collections of monocytic cells about vessels were seen. Some of the tubules were lined with basophilic cells and mitosis was occasionally observed. The nuclei of the tubular cells varied considerably in size and staining quality. With repeated subcutaneous doses varying from 2-50 units per 20 g mouse, renal lesions observed one week after the final injection were inconspicuous, consisting for the most part of occasional tubular casts. Necrotic tubules were infrequent.

The thyroids appeared moderately hyperplastic.

In the rat renal lesions were minimal and necrosis of cells rare.

Other pharmacological data. The best available preparation of bacitracin (No. B-100) was used in gathering the following data. A 5% solution containing 1500 units per cc was found, by means of the freezing point depression, to be approximately isotonic with frog-Ringer solution. The contractility and rhythmicity of the frog's heart perfused by the Straub technic were not altered by the addition of 15 units of bacitracin per cc of frog-Ringer solution. Higher concentrations often caused diminished contractility and progressive slowing.

Tests with the isolated guinea pig ileum indicated that bacitracin contains a minute amount of a histamine-like substance; for example, 100 mg or 3,000 units of preparation B-100 contained the equivalent of approximately 3 μ g of histamine diphosphate. Bacitracin-induced contractions were essentially abolished by first adding 1 cc of 1-100,000 pyribenzamine hydrochloride to

the 50 cc bath used in the experiments.

Intravenous injection of bacitracin solution into the dog, anesthetized by intravenous pentobarbital, caused an acute transient fall of blood pressure immediately followed by an equally transient rise; for example, 10 mg or 300 units of lot B-100 per kg body weight caused a fall of 30-40 mm of Hg followed by a rise of 10-20 mm of Hg above the pre-injection level of the blood pressure. Each effect lasted only a few seconds. Either benadryl or pyribenzamine essentially abolished the action of equidepressor doses of histamine diphosphate, but neither altered the depressor effect of bacitracin. Hence, histamine is not the cause of the depressor effect. This depressor effect, unlike that of acetylcholine, was not prevented by atropine sulfate. The pressor phase of the diphasic blood pressure response and the vagal heart beats associated with it disappeared after the injection of pyribenzamine.

Summary. The acute, intraperitoneal toxicity of bacitracin concentrates was greater in rats than in mice. The subcutaneous LD_{50} in mice was 4-7 times the intraperitoneal LD_{50} . Lethal results were produced only with immense oral doses. Damage to the renal tubules, with tubular necrosis after very large doses, was observed in mice whereas in rats there was little kidney involvement. The toxicity of bacitracin, as measured by the LD_{50} , appeared to be independent of the antibiotic activity. Concentrates of the antibiotic contained a minute amount of an histamine-like substance which was not responsible for the definite depressor effect caused by the intravenous injection of 10 mg (300 units) per kg into the dog.

We are indebted to Professor Harry B. van Dyke for his kind interest and active participation in this work.

Pathogenicity of the Tubercle Bacillus.*

SIDNEY RAFFEL.[†]*From the Department of Bacteriology and Experimental Pathology, School of Medicine, Stanford University, California.*

The factors responsible for the ability of *Mycobacterium tuberculosis* to produce disease in a susceptible host have long been enigmatic. The criteria commonly employed to judge the probable virulence of many other pathogenic bacteria have not been applicable in this instance. For example, although dissociation occurs in cultures of tubercle bacilli,¹ smoothness or roughness of colonial form does not provide a satisfactory basis for predicting the activity of this organism in the tissues of a susceptible species of host.² It is mystifying indeed that an attenuated bacterium such as the B.C.G. (*Bacillus Calmette-Guérin*) should possess, so far as we know, all the properties of the virulent tubercle bacillus except the ability to produce a progressive generalized infection. At the site of injection it induces the typical tuberculous lesion, and from this area viable bacilli may be cultured after many months.

It is difficult to deduce from available information whether the virulent tubercle bacillus possesses some cytoplasmic constituent analogous to the somatic antigens associated with virulence in many other bacterial species. One of the major functions of such substances in permitting organisms to multiply in tissues is the phagocyte-repellent effect. No such effect is apparent in the case of *M. tuberculosis*, for virulent as well as attenuated organisms are readily taken up by monocyctic phagocytes. Its pathogenicity may conceivably depend upon a metabolic

feature of the bacterial cell, the virulent organism being better able to propagate in the environment supplied by viable tissues. For this view also evidence is lacking.

Should virulence depend in part upon a specific cellular antigen, it seems a rational probability that the acquisition of immunity will also be related to this substance, for in the final analysis acquired resistance means the ability of the body specifically to "neutralize" the virulence mechanism of the bacterium. A familiar case in point is the protection provided by the antibody directed against the capsular S.S.S. of *D. pneumoniae*. If, on the other hand, virulent tubercle bacilli are distinguished by a metabolic trait which favors their growth in the tissues, then it is quite possible that no ordinary immunologic device with which we are familiar will divulge the nature of acquired resistance to them.

Several years ago we undertook a study designed to reveal whether some one antigenic component of *M. tuberculosis* may be responsible for inducing acquired resistance, and as a corollary, whether this factor would enable us to demonstrate, on an immunologic basis, the dissociation or identity of resistance and allergy in this disease.³

Attempts to induce the resistant state in the absence of allergy have so far been unsuccessful. A good deal of attention has been devoted to the polysaccharide of the bacillus in this connection. This substance however is haptenic, and its reconversion to a complete antigen has not proved satisfactory, so that a fair test of its immunizing ability has not been possible. However, we have found that when this substance was injected daily in amounts up to 15 mg to guinea pigs throughout a 10-week course of immunization with B.C.G., and then for 20

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¹ Petroff, S. A., and Steenken, W., Jr., *J. Exp. Med.*, 1930, **51**, 831.

² Smithburn, K. C., *Am. Rev. Tuberc.*, 1937, **36**, 637.

³ Raffel, S., *Stanford Med. Bull.*, 1943, **1**, 209.

weeks subsequent to infection with virulent organisms ($H_{37}Rv$), it failed to antagonize the acquired resistance produced by the B.C.G.⁴ It appears then that acquired immunity to *M. tuberculosis* is not related in any major degree to an antibody type of response to this particular bacillary antigen. The purified phosphatide, the wax, and the protein have also been employed as vaccinating materials, and these have failed to induce resistance to the bacillus of origin.⁵

More success has attended our studies of the possible relationship of tuberculous allergy to resistance. It has been appreciated for many years that this hypersensitive state is a response to the protein of the organism. Peculiarly enough, however, the tuberculin-type of allergy cannot be initiated by the use of the bacillary protein in isolated form, although the substance has marked antigenic properties and on injection into normal animals regularly induces humoral antibodies and the anaphylactic state. We have found that the delayed type of tuberculin hypersensitivity can be established in the absence of tubercle bacilli by employing the chloroform extractable "wax" of the bacillus. Such wax contains a small proportion of protein, and when injected into normal guinea pigs it regularly establishes good levels of delayed reactivity to Old Tuberculin.⁵ The application of several criteria for the tuberculin-type of hypersensitivity, including vulnerability of such sensitized tissues to tuberculin in tissue culture, and failure to transfer the sensitivity passively via serum, have demonstrated that this is true tuberculous allergy. Tests on a limited number of guinea pigs have indicated that animals rendered hypersensitive by this means do not possess acquired resistance to tuberculous infection. This observation has convinced us that allergy and acquired immunity are immunologically distinguishable.

Experimental. Our more recent efforts to isolate a resistance-inducing factor from the bacillus has led us to investigate antigens of the "Boivin type."⁶ These are complex

substances composed of polysaccharide, lipid and protein, and have been obtained from a variety of the Gram-negative enteric bacilli. They are of utmost immunological importance in such organisms, since they comprise the essential immunizing antigen and also reveal the toxic properties commonly associated with these bacteria. Choucroun^{7,8} has reported the presence of a toxic fraction in paraffin oil extracts of tubercle bacilli which apparently induces acquired resistance. This is a polysaccharide ester of mycolic acid, and may be related to the Boivin type of antigen.

Since Boivin's original demonstration of the extractability of antigenic complexes by trichloroacetic acid,⁶ several other methods have been described for accomplishing this end. Because we had no foreknowledge of the relative susceptibility of the tubercle bacillus to these extraction methods, we decided to employ them all on a comparative basis. The procedure of Raistrick and Topley⁹ makes use of trypsin digestion; Morgan¹⁰ employed diethylene glycol, and Walker¹¹ used urea for this purpose.

Preparation of Complexes. Human $H_{37}Rv$ bacilli were cultured on a modified Long-Seibert synthetic medium. At the end of 3 weeks the organisms were separated from the medium and washed with distilled water. A portion of these bacilli was washed in 50%, and then in absolute acetone in a refrigerated centrifuge. Immediately afterward the bacilli were dried in a vacuum dessicator over sulphuric acid. The remainder were employed in the fresh moist state. This difference in treatment was instigated by uncertainty as to the effect of even a short period of acetone treatment upon the organisms in view of the presence of acetone soluble fats in tubercle bacilli. Batches of the cells were extracted as shown in Table I, where the

⁶ Boivin, A., Mesrobian, L., Mesrobian, L., and Nestorescu, B., *C. R. Soc. Biol.*, 1934, **115**, 306.

⁷ Choucroun, N., *Science*, 1943, **98**, 327.

⁸ Choucroun, N., *Science*, 1947, **105**, 46.

⁹ Raistrick, H., and Topley, W. W. C., *Brit. J. Exp. Path.*, 1934, **15**, 113.

¹⁰ Morgan, W. T. J., *Biochem. J.*, 1937, **31**, 2003.

¹¹ Walker, J., *Biochem. J.*, 1940, **34**, 325.

⁴ Unpublished observations.

⁵ Raffel, S., *Am. Rev. Tuberc.*, 1946, **54**, 54.

TABLE I.
Essential Information Concerning the Extraction of Complexes from Tubercle Bacilli.

Bacillary batch No.	Treatment prior to extraction	Grams of bacilli extracted	Extraction method	Mg of lyophilized complex obtained
1	Washed with water, acetone dried	15.6 (dry wt)	Boivin—trichloroacetic acid	219.0
2	Washed with water	10 (moist wt)	" "	50.0
3	Washed with water, acetone dried	15.9 (dry wt)	Raistrick and Topley—trypsin digestion	257.0
4	Washed with water	25.2 (moist wt)	Walker—urea	51.4
5	Washed with water, acetone dried	15.4 (dry wt)	Morgan—diethylene glycol	26.5

yields of the individual complexes are also indicated. All of the complexes were lyophilized and stored in a carbon dioxide box. They formed colloidal suspensions when dissolved in saline or water.

During the course of their preparation these substances were subjected to the following physical manipulations calculated to free the extracts of intact bacilli:

The 2 trichloroacetic acid preparations were freed of most bacteria by centrifugation in the cold. The supernatants were then ultracentrifuged for 15 minutes at 12,000 r.p.m. Following this the extracts were passed through Jena 5/3 glass filters, and were then lyophilized. The trypsin and urea preparations were freed of most of the organisms by Sharples centrifugation. These fluids were filtered through Chamberland L₃ candles prior to lyophilization.

The diethylene glycol extract, because of its smaller volume and the nature of the fluid, was spun in the ultracentrifuge at 25,000 r.p.m. for one hour. This material was subsequently filtered through a Chamberland L₃ candle also.

Treatment of guinea pigs. These lyophilized preparations were freshly dissolved for each immunizing injection. Fifty-three guinea pigs weighing between 400 and 500 g, and negative in skin test to Old Tuberculin 1:10, were given the first injections of these complexes on August 19, 1946. Ten or 11 animals were apportioned to each of the preparations. These groups of guinea pigs will be referred to by the numbers used to

designate the bacillary batches in Table I. The dose of each complex administered was 0.5 mg in 0.25 cc of saline, injected subcutaneously. Three weeks later skin tests with O.T. 1:10 proved negative in all groups. One month following the first injection, second injections of the same dose of the respective complexes were administered, this time intracutaneously. This was repeated a month later, subcutaneously. Thus, during a period of 8 weeks these animals received 3 doses of complex, totaling 1.5 mg for each animal.

Twelve weeks after the first injection of each complex, routine skin tests were carried out with undiluted O.T. and the results were quite unexpected. Every one of the 43 animals comprising Groups 1, 2, 3 and 4 developed exceedingly large necrotizing reactions, averaging 29 mm in diameter. These were unusually severe in that practically the entire areas were necrotic within 24 hours. One week later tests were repeated in 12 of these guinea pigs with less concentrated O.T. (1:10). The average diameter of reaction was 25 mm, and in 10 of these necrosis occurred. None of the animals receiving the diethylene glycol complex developed sensitivity to O.T., either at this time nor during several succeeding months of injections.

One of the animals of Group 4 (urea complex) which died as a result of the initial tuberculin reaction, showed marked tuberculosis involving the axillary lymph nodes and the spleen. Histologic examination revealed lesions with bacilli in these locations. All of the remaining animals in Groups 1,

TABLE II.
Gross Tuberculosis in Regional Lymph Nodes, Abdominal and Thoracic Viscera of Guinea Pigs
Receiving Injections of Complexes.

Guinea pig group	No. of pigs in groups	No. showing involvement of lymph nodes regional to inject. (axillary and ing.)	No. with involvement of liver and/or spleen	No. with involvement of lungs and/or tracheobronchial lymph nodes
Trichlor. extract of dried bacilli	9	8	9	7
Trichlor. extr. of fresh bacilli	9	8	8	6
Trypsin extr. dried bacilli	11	8	6	4
Urea extr. fresh bacilli	8	7	7	5
Total	37	31	30	22

2, 3 and 4 were subsequently examined at autopsy for the degree and extent of dissemination of tuberculosis. The overall results are shown in Table II. Thirty-one of the 37 animals showed gross evidence of infection at 3 months or later following the first injection of the respective complexes. The animals which failed to reveal such evidence had previously responded to Old Tuberculin with skin reactions equal in severity to those seen in the pigs with obvious disease. Of the 31 animals, most showed advanced infection, and a few were striking in this regard. As indicated in Table II infection was most evident in the abdominal viscera and in the axillary and inguinal lymph nodes draining the areas of injections of the complexes, while a smaller proportion of animals showed disease also in the lungs or tracheobronchial lymph nodes. In no case was there infection in the lungs or tracheobronchial nodes in the absence of abdominal visceral or axillary-inguinal node involvement. It is thus rather apparent that infection was consequent to the injections, and was not spontaneous as the result of aspiration of bacilli from the environment. Added to this evidence is the fact that groups of normal unused guinea pigs as well as those receiving other, noninfectious vaccinating treatments such as the diethylene glycol complex and various preparations of proteins derived from the tubercle bacillus, were housed in the same large room during the entire period of this experiment. These have never responded to repeated tests with concentrated Old Tuberculin, nor has evidence of tuberculosis been encountered in autopsies of many

such animals. Furthermore, it has been a general experience as well as our own that guinea pigs are very unreliable to spontaneous infection with *M. tuberculosis* even after intimate and prolonged contacts.

Since now it was apparent that the complexes which had been intended as immunizing agents were actually infectious, we undertook to determine what numbers of bacilli could have escaped the centrifugation and filtration procedures employed in the preparation of these substances. For this purpose the total residual lyophilized complexes were dissolved in saline and ultracentrifuged at 25,000 r.p.m. for one hour. No visible sediments were thrown down, but the bottoms of the tubes were carefully scraped and smears made of the scrapings. These were examined for acid-fast bacilli. As shown in Table III, very few tubercle bacilli were encountered, although in every case the sediments represented amounts of complex greatly in excess of that received by the animals.

TABLE III.
Results of Search for Tubercle Bacilli in the
Ultracentrifugal Sediments of Complexes.

Complex	Mg of complex ultra- centrifuged	Result of exam- ination of total recoverable sediment
1. Trichloroacetic acid extr. of dried bacilli	200	6 bacilli
2. Trichloroacetic acid extr. fresh bacilli	35	2 possible bacilli, not distinct
3. Trypsin digest extr. dried bacilli	240	4 bacilli
4. Urea extr. fresh bacilli	35	8 "

It was now evident that these guinea pigs had developed a surprising incidence and extent of infection as the result of the very small numbers of tubercle bacilli which they had received. Since we had made frequent use of this strain of organisms (H₃₇Rv) for infecting guinea pigs in the past, we were able to gather from previous records the relative degrees of tuberculosis established at various comparable intervals following infection with measured doses of bacteria. Guinea pigs which had received 2 ranges of infective doses by the subcutaneous route were reviewed for this purpose. The first group had been infected with 0.75 or 1.0 mg (moist weight) of bacilli, and the second had received doses of 0.05, 0.075 or 0.10 mg. A third group of pigs was available for a much more direct and striking comparison. These animals had received acetone-dried bacilli of the same batch employed for the preparation of the trichloroacetic extract complex (No. 1, Table I) and the trypsin complex (No. 3, Table I). These bacilli failed to grow on glycerine egg medium during an 8-week period of observation. The organisms were administered to 13 guinea pigs in doses of 10 mg dry weight for each subcutaneous injection. A total of 40 mg was given to each animal in 4 injections during a period of 3 months.

In order to compare these 3 groups with the animals receiving complexes, the Feldman index¹² was employed to score the gross tuberculosis observed at autopsy. This index takes account of the extent of involvement of the lymph nodes draining the region of inoculation, the spleen, liver, lungs and the tracheobronchial lymph nodes.

The average indices for each group of animals are portrayed in Table IV and Fig. 1. It may be noted that the averages of organ involvement in the animals receiving complexes were uniformly greater at the various examination intervals than those observed in the guinea pigs injected with massive doses of acetone dried bacilli, and were not far below those of the animals infected with the

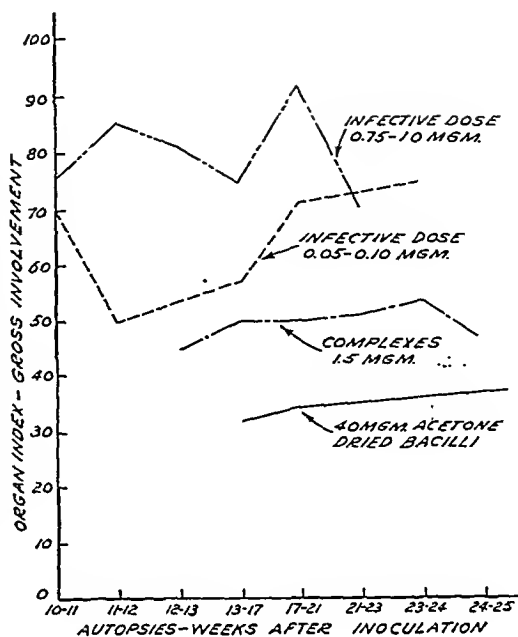


Fig. 1.

0.05 to 0.10 mg doses of fresh young culture organisms. The guinea pigs which had received 0.75 to 1.0 mg amounts of bacilli to induce infection showed more definitely advanced disease at comparable intervals following infection.

Discussion. The factors which should enter into consideration of these results are the following:

(a) The animals of the "complex groups" received a total of 1.5 mg of material of which much greater amounts revealed sparse tubercle bacilli in ultracentrifugal sediments. In contrast, the groups of guinea pigs included in the table for purposes of comparison received relatively enormous numbers of bacteria of the same strain, and in the case of the acetone dried organisms, of the same batch and at the same time.

(b) The bacilli which remained in the complexes had been subjected to chemical and physical procedures as well as aging in the lyophilized state. The organisms employed for infecting 2 of the comparative groups of animals were from 2-week-old cultures, freshly suspended in saline immediately be-

¹² Feldman, W. H., *Am. Rev. Tuberc.*, 1943, **48**, 248.

TABLE IV.
Scoring of Guinea Pig Organs for Extent of Grossly Observable Tuberculosis at Various Periods After Infection.

Wks after infection	Infected with							
	0.75 or 1.0 mg fresh bacilli		0.05-0.10 mg fresh bacilli		40 mg acetone dried bacilli*		bacilli present in complexes†	
	No. of G pigs	Avg score	No. of G pigs	Avg score	No. of G pigs	Avg score	No. of G pigs	Avg score
10-11	23	76	2	70				
11-12	8	86	2	50				
12-13	7	81					1	45
13-17	12	74	7	58	2	32	9	50
17-21	2	92	8	71	8	34	2	50
21-23	1	70					4	51
23-24			1	75			10	53
24-25							11	47
26-32					3	38		

* Infection interval dated from 1st injection of 10.0 mg of bacilli. A total of 40 mg was eventually injected into all pigs.

† Infection interval dated from 1st injection of 0.50 mg of the various complexes. A total of 1.5 mg was eventually injected into all pigs.

fore use. The acetone-dried bacilli given to the third comparative group had been subjected to considerably less manipulation than was accorded those organisms remaining in the complexes.

(c) The infection intervals in the "complex animals" are dated from the time of first injection of these substances. The 3 injections actually spanned a period of 8 weeks, and with the small numbers of organisms involved, some of the animals may well have received no bacilli on the first, and perhaps the second, injection. The intervals after infections set forth in Table II may therefore be considerably exaggerated in some of these animals.

It appears then that the ability of the very sparse numbers of tubercle bacilli present in the complexes to produce a degree of infection greater than that found following massive doses of similarly treated organisms alone must depend upon some function of the complexes themselves. So far as can be deduced

from these experiments, the complexes acted in the capacity of a virulence factor, permitting small numbers of possibly "injured" bacilli to establish themselves in the normal host and to produce progressive infection.

Whether such complexes can function in the same capacity in relation to attenuated bacilli, such as B.C.G., is being investigated at the present time. The possible role of these substances in inducing acquired resistance is also under study.

Conclusions. The Boivin type of antigenic complexes derived from the human strain ($H_{37}Rv$) of *M. tuberculosis* appear to abet generalized infection in guinea pigs receiving very small numbers of organisms. It is suggested that such complexes may function as a "virulence factor" for the tubercle bacillus in a susceptible species of host. The possible relationship of such a factor to acquired resistance to the tubercle bacillus is under investigation.

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